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Novel Regulators of Vitamin D Action and Metabolism: Lessons Learned at the Los Angeles Zoo

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Abstract We undertook an investigation of an outbreak of rachitic bone disease in the Emperor Tamarin New World primate colony at the Los Angeles Zoo in the mid-1980s. The disease phenotype resembled that observed in humans with an inactivating mutation of the vitamin D receptor (VDR), hypocalcemia, high 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D) levels, and rickets in rapidly growing adolescent primates. In contrast to the human disease, the New World primate VDR was functionally normal in all respects. The proximate cause of vitamin D hormone resistance in New World primates was determined to be the constitutive overexpression of a heterogeneous nuclear ribonucleoprotein in the A family which we coined the vitamin D response element binding protein (VDRE-BP). VDRE-BP competed *in trans* with the VDR-retinoid X receptor (RXR) for binding to the vitamin D response element. VDRE-BP-legislated resistance to 1,25-$(OH)_2$D was antagonized (i.e., compensated) by another set of constitutively overexpressed proteins, the hsp-70-related intracellular vitamin D binding proteins (IDBPs). IDBPs, present but expressed at much lower levels in Old World primates including man, exhibited a high capacity for 25-hydroxylated vitamin D metabolites and functioned to traffic vitamin Ds to specific intracellular destinations to promote their action and metabolism. J. Cell. Biochem. 88: 308–314, 2003.

Key words: primate; vitamin D resistance; heterogeneous nuclear ribonuclear protein; heat shock protein; rickets

INTRODUCTION TO PRIMATE EVOLUTION

An understanding of the work to follow will require a few comments on the process of early primate evolution. In the Eocene period, some 50–100 million years ago, the great southern hemispheric landmass, Pangea, broke apart with the Americas and Madagascar moving away from Africa. This continental separation occurred early in the process of primate evolution, trapping primordial primates in South America, Africa, and Madagascar. From here, the three major primate infraorders, platyrrhines, or New World primates, the catarrhines or Old World primates, and lemurs, evolved independently of one another [Pilbeam, 1984]. Compared to Old World primates, including our own species, New World primates are confined to Central and South America. They are generally smaller in stature than Old World primates, a characteristic that is well suited to their lifestyle as plant-eating, arboreal sunbathers residing in the canopy of the periequatorial rain forests of the Americas.

OUTBREAK OF RICKETS IN THE NEW WORLD PRIMATE COLONIES OF THE LOS ANGELES ZOO

The index case in our original studies was a pre-adolescent New World primate of the Emperor tamarin species. When investigated radiographically, this and related tamarins and marmosets displayed classical rickets complete with growth retardation and metaphyseal cupping and fraying. In order to investigate this rachitic syndrome, we collected blood and urine from involved monkeys as well as from control, nonrachitic New- and Old World primates. That comparison yielded a biochemical phenotype (Fig. 1A) that was most remarkable for an elevated serum 1,25-dihydroxyvitamin D

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A

Rachitic Phenotype

blood calcium.....	slightly decreased
urine calcium	slightly decreased
blood phosphate.....	normal
urine phosphate	normal
serum creatinine.....	normal
liver function.....	normal
25-OHD.....	normal
1,25-(OH)2D.....	very high

B

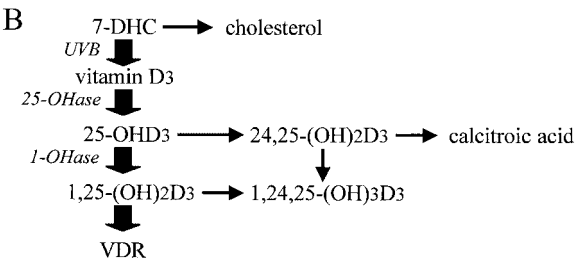

Fig. 1. Biochemical phenotype of rachitic New World primates. **Panel A** demonstrates biochemical indices of bone health in New World primate suffering from rickets compared to developmental age- and sex-matched nonrachitic Old World primates. The outstanding characteristic is a 1,25-dihydroxyvitamin D (1,25-$(OH)_2D$) level 2 orders of magnitude greater than that observed in Old World primates, including man. The bold arrows in the simplified vitamin D synthetic/metabolic scheme in **panel B** describe the means by which these high 1,25-$(OH)_2D$ are achieved and maintained. Ultraviolet B photons (*UVB*) exposure is increased in the natural habitat of New World primates, the canopy of the equatorial rain forests of Central and South America. Increased cutaneous vitamin D_3 synthesis results in increased production, via the hepatic vitamin D-25-hydroxylase (*25-OHase*), and serum levels of 25-hydroxyvitamin D_3 (25-OHD_3). Elevated 1,25-$(OH)_2D_3$ levels are achieved in part by increased synthesis of the hormone via the 25-hydroxyvitamin D-1-hydroxylase (*1-OHase*). 1,25-$(OH)_2D_3$ is then available to the VDR in relatively large quantities to compensate for the hormone-resistant state characteristic of New World primates.

(1,25-$(OH)_2D$) level in rachitic New World primates [Adams et al., 1985a]. In fact, with the exception of nocturnal primates in the genus Aotus, New World primates in all other genera had vitamin D hormone levels ranging up to two orders of magnitude higher than that observed in Old World primates including man [Adams et al., 1985a; Adams et al., 1987; Gacad and Adams, 1991].

As it turned out, New World primates with rickets were those with the lowest 1,25-$(OH)_2D$ levels, while their healthy counterparts were those with the highest serum 1,25-$(OH)_2D$ levels. These data were interpreted to mean that most New World primate genera were naturally resistant to the vitamin D hormone, and that the resistant state could be compensated by maintenance of high 1,25-$(OH)_2D$ levels. That turned out to be the case. When rachitic New World primates were exposured to 6 months of artificial sunlight in their enclosures, both substrate serum 25-hydroxyvitamin D (25-OHD) and product 1,25-$(OH)_2D$ levels rose dramatically resulting in cure of rickets [Gacad and Adams, 1992a].

In summary, New World primates are periequatorial sunbathers for a reason. As depicted by the oversized arrows in a simplified scheme of vitamin D synthesis and metabolism (Fig. 1B), New World primates require a great deal of cutaneous vitamin D synthesis in order to push their 25-OHD and 1,25-$(OH)_2D$ levels high enough to effectively interact with the VDR. The question remains as to why these primates are resistant to all but the highest levels of the vitamin D hormone?

INVESTIGATING THE BIOCHEMICAL NATURE OF VITAMIN D RESISTANCE IN NEW WORLD PRIMATES

In order to answer the above question, cultured fibroblasts and immortalized cell lines from both resistant and hormone-responsive New and Old World primates were used to track, step by step, the path taken by the vitamin D hormone from the serum vitamin D binding protein (DBP) in the blood in route to the nucleus and transactivation of hormone-responsive genes [Adams et al., 1985a; Adams et al., 1985b; Adams and Gacad, 1988; Gacad and Adams, 1991, 1992a,b, 1993; Arbelle et al., 1996; Chen et al., 1997, 2001] (Fig. 2B). We discovered that the movement of hormone from DBP across the cell membrane and through the cell cytoplasm and nuclear membrane in New World primate cells was indistinguishable from that observed in Old World primate cells. We also determined that the ability of the New World primate VDR to bind to 1,25-$(OH)_2D_3$ and induce receptor dimerization with the RXR was normal. In fact, when removed from the intranuclear environment, the VDR in New World primates was akin to the Old World primate receptor in all respects [Chun et al., 2001]. That which was not the same in New World primate cells was the reduced ability of

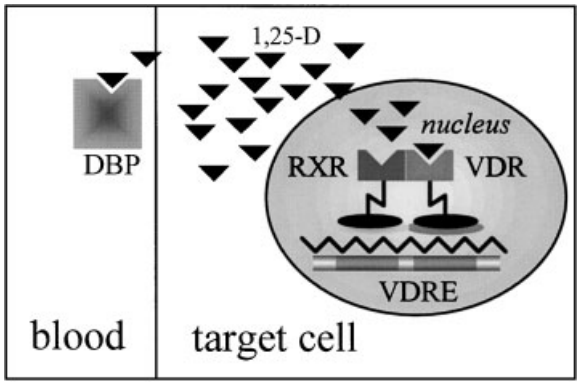

Fig. 2. Pathway of hormone 1,25-dihydroxyvitamin D (1,25-D) from the blood to nucleus of the target cell in the vitamin D-resistant New World primate. Compared to the Old World primate scenario, vitamin D hormone moves normally from the circulating vitamin D binding protein (DBP), through the cell membrane and cytoplasm and onto the vitamin D receptor (VDR). Once liganded, the VDR paired with the retinoid X receptor (RXR) can move as a heterodimeric complex to the cell nucleus. The jagged line at the vitamin D response element (VDRE) represents a relative inability in the New World primate cell of the heterodimeric receptor complex to engage the *cis* element. This and the accumulation of hormone in the cell cytoplasm represent salient disparities in hormone handling and action in the New World primate compared to the Old World primate cell.

VDR–RXR complex to bind to its cognate *cis* element and transact. In addition to this failure was the apparent build-up of hormone in the cytoplasm.

In order to elucidate nuclear receptor events in New World primate cells, we isolated and extracted the nuclei of New World primate cells. We found that these extracts contained a protein, in addition to the VDR–RXR, that was bound by the VDRE. We coined this the vitamin D response element binding protein or VDRE-BP [Chen et al., 2000]. In electromobility shift assays (EMSA) using the VDRE as probe, Old World primate cell extract contained only the VDR–RXR bound to the VDRE probe, while the New World primate extract contained two probe-reactive bands, one compatible with the VDR–RXR and a second, more pronounced VDRE-BP-VDRE band (Fig. 3A). This VDRE-BP-VDRE binding reaction was specific as the VDRE-BP could be competed away from VDRE probe by the addition of excess unlabelled VDRE. These data suggested that VDRE-BP might function as a dominant-negative inhibitor of receptor-response element binding by competing *in trans* with receptor, "knocking it off" the VDRE. That was the case. In EMSAs where recombinant human VDR and RXR were

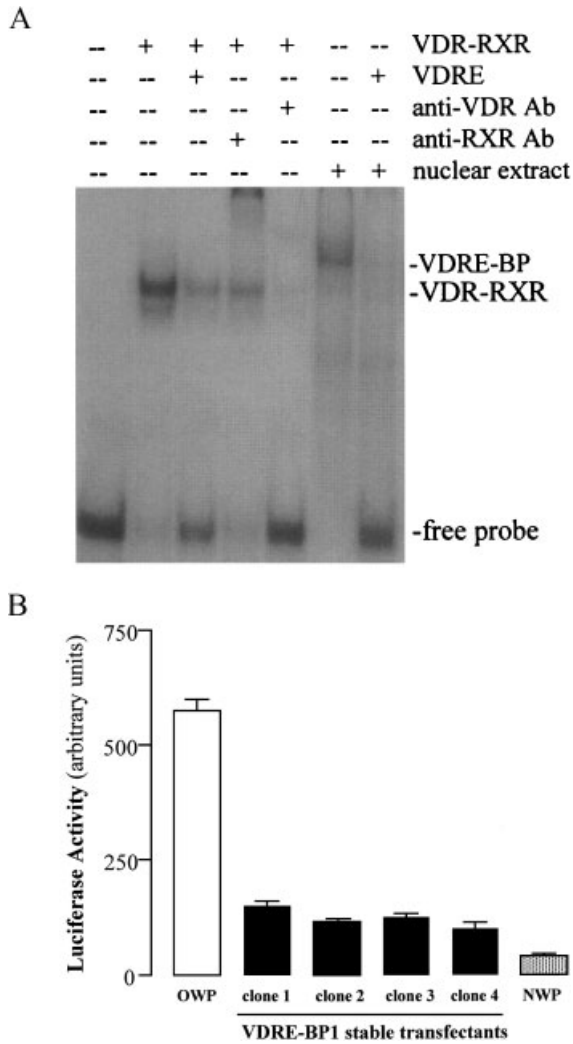

Fig. 3. Evidence for the dominant-negative action of the New World primate vitamin D response element binding protein (VDRE-BP). **Panel A** is an electromobilty shift assay, using consensus vitamin D response element as probe, showing the presence of a second *trans* binding protein, in addition to the vitamin D receptor (VDR)–retinoid X receptor (RXR), in nuclear extracts of vitamin D-resistant New World primate cells. Addition of excess unlabelled VDRE is shown in **lanes 3** and **7**. Addition of anti-RXR*alpha* antibody (**lane 4**) supershifts while addition of either anti-VDR antibody (**lane 5**) or New World primate nuclear extract containing the VDRE-BP (**lane 6**) competes away probe-VDR-RXR binding. **Panel B** demonstrates significant squelching ($P < 0.001$) of VDR–RXR-directed, VDRE-reporter-driven transactivation in four different clones of Old World primate (OWP) cells after stable transfection with the New World primate vitamin D response element binding protein-1 (VDRE-BP1); reporter activity in untransfected New World primate (NWP) cells is shown for comparison. These data are reprinted with permission of the authors.

permitted to interact with increasing amounts of nuclear extract from either vitamin D-resistant cells containing a VDRE-BP or from normal vitamin D-responsive cells, the addition

of more control extract acted only to amplify the VDR-RXR-retarded probe on the gel. By contrast, increasing amounts of the hormone-resistant extract competed away VDR-RXR-probe binding in favor VDRE-BP-probe binding.

VITAMIN D RESPONSE ELEMENT BINDING PROTEINS

To date we have identified three distinct VDRE-BPs, two in New World primates and one in humans. They are all members of the heterogeneous nuclear ribonucleoprotein A family [Chen et al., 2000], previously considered to be only single strand mRNA binding proteins [Dreyfuss et al., 1993]. However, as just mentioned, VDRE-BPs can also bind specifically to double strand DNA. In fact, it is by virtue of their ability to bind DNA that they can be distinguished from traditional corepressor proteins [Horwitz et al., 1996]. When overexpressed, they can effectively squelch VDR-directed transactivation. This ability to squelch transactivation is shown in Figure 3B. Depicted is VDRE-directed reporter activity in four different subclones of wild-type Old World primate cells stably overexpressing the New World primate VDRE-BP-1 and in naturally hormone-resistant New World primate cells. In all instances, stable overexpression of VDRE-BP-1 squelched VDRE-directed luciferase activity substantially compared to the untransfected, wild-type host cell to the levels observed in hormone-resistant New World cells that naturally overexpress the protein. We take this as strong confirmatory evidence that, when naturally overexpressed in vivo as one or more of them are in New World primates, these proteins are the cause vitamin D resistance in these monkeys.

INTRACELLULAR VITAMIN D BINDING PROTEINS

On our way to the discovery of the VDRE-BPs in New World primate cells, we also observed that these cells were extraordinarily efficient at accumulating 25-hydroxylated vitamin D metabolites in the cytoplasmic space (Fig. 2B). Accumulation here was the result of expression of a second set of resistance-associated proteins. These intracellular vitamin D binding proteins [Gacad et al., 1997; Gacad and Adams, 1998], or IDBPs as they have come to be called, exhibit both high capacity and high affinity for 25-hydroxylated vitamin D metabolites. In fact, among all of the vitamin D metabolites that have been tested, IDBP purified from vitamin D resistant New World primate cells binds 25-OHD_3 and 25-OHD_2 best [Gacad and Adams, 1991; Gacad and Adams, 1998]; in a competitive displacement assay using radioinert 25-OHD_3 as competitor and [^{3}H] 25-OHD_3 as labeled ligand the concentration of metabolite required to achieve half-maximal displacement of labeled hormone (EC_{50}) was <1 nM. Although normally present in Old World primate including human cells, these proteins can be over-expressed some 50-fold in New World primate cells. They are highly homologous to proteins in the heat shock protein-70 family [Gacad et al., 1997]. The first three members of this family cloned and characterized by us, IDBP-1, -2, and -3, bear a high degree of sequence identity with constitutively-expressed human heat shock protein-70 (hsc 70), heat shock inducible heat-shock protein-70 (hsp 70), and mitochondrial-targeted grp-75, respectively. They all contain an ATP-binding-ATPase domain ahead of a protein–protein interaction domain [Hartl, 1996]. Some, like IDBP-3, also harbor an N-terminal organelle-targeting domain. Preliminary studies indicate that the vitamin D ligand binding domain is in the middle of the molecule [Wu et al., 2000].

What are these IDBPs doing inside the hormone-resistant New World primate cell? We considered two countervailing hypotheses to explain the function of these proteins. One hypothesis held that these IDBPs were "sink" molecules that worked in cooperation with the VDRE-BP in the nucleus to exert vitamin D resistance by disallowing access of the hormone to the VDR and the nucleus of the cell. The opposing hypothesis held that these were "swim" molecules that actually promoted the delivery of ligand to the vitamin D receptor, improving the ability of the VDR to dimerize and bind to DNA, antagonizing the actions of the VDRE-BP that was overexpressed in New World primate cells. In order to determine which of these hypotheses was correct, we stably overexpressed the most abundant of these IDBPs, IDBP-1, in wild-type Old World primate cells and demonstrated that IDBP-1 imparted protransactivating potential [Wu et al., 2000]; the endogenous transcriptional activity of three different 1,25-$(OH)_2$D-responsive genes, the vitamin D-24-hydroxylase, osteopontin and osteocalcin, in Old World primate wild-type cells was markedly enhanced

(Fig. 4A). We concluded from these studies, at least for the function of transactivation, that IDBP-1 was a "swim" molecule for the vitamin D hormone, promoting delivery of ligand to the VDR.

Considering the facts that New World primates are required to maintain very high serum levels of 1,25-$(OH)_2$D in order to avert rickets (Fig. 1B), we also hypothesized that the IDBPs, which are known to bind 25-OHD even better than 1,25-$(OH)_2$D, will also promote the synthesis of the active vitamin D metabolite via promotion of the 25-OHD-1-hydroxylase. Evidence that this is the case is provided in Figure 4B. When human kidney cells expressing the 25-OHD-1-hydroxylase gene were stably transfected with IDBP-1 and incubated with substrate 25-OHD_3, 1,25-$(OH)_2D_3$ production went up 4–8-fold compared to untransfected wild-type cells [Wu et al., 2002]. This increase in specific 25-OHD-1-hydroxylase activity occurred independently of a change in expression of the 25-OHD-1-hydroxylase gene [Wu et al., 2002]. In fact, current data [R. Chun, unpublished] now strongly indicate that this increase in hormone production is the result of the ability of IDBPs to promote the delivery of substrate 25-OHD to the inner mitochondrial membrane and the 25-OHD-1-hydroxylase stabled there.

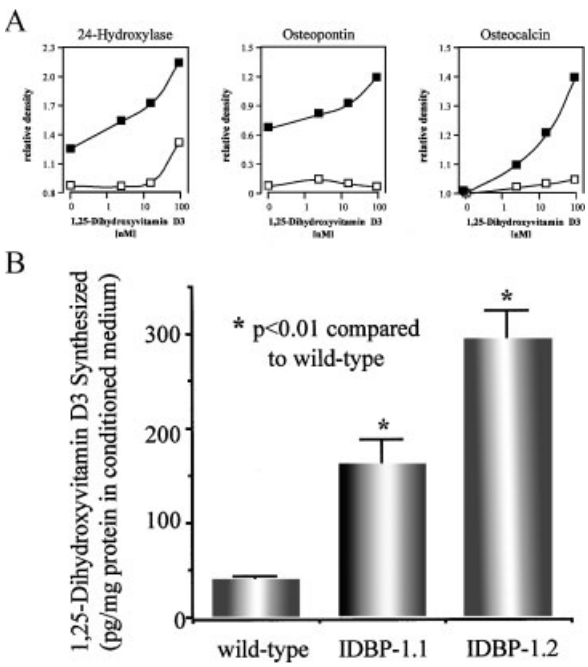

Fig. 4. Consequences of stable overexpression of members of the New World primate intracellular vitamin D binding protein (IDBP) family in vitamin D-responsive Old World primate cells. **Panel A** depicts the 1,25-dihydroxyvitamin D concentration-dependent increase in the endogenous expression level by Northern blot analysis of three hormone-responsive genes in Old World primate cells before (open squares) and after (closed squares) stable overexpression of IDBP-1. **Panel B** demonstrates the 1,25-dihydroxyvitamin D synthetic capacity of Old World primate (wild-type) cells before and after and stable overexpression of IDBP-1; IDBP-1.1 and -1.2 represent different clones of cells stably-transfected with IDBP-1. These data are reprinted with permission of the authors.

A NEW MODEL FOR INTRACELLULAR VITAMIN D TRAFFICKING

Dogma has held that sterol/steroid hormones like vitamin D, by nature of their lipid solubility, move through the plasma membrane of the target cell and "ping-pong" around the cell interior until they encounter another specific binding protein like the 25-OHD-1-hydroxylase or the VDR with which to bind. Our most recent results, developed from a compendium of confocal imaging studies with fluorescently-labeled IDBPs and vitamin D metabolites as well as with gst "pull-down", co-immunoprecipitation and yeast 2-hybrid binding experiments [R. Chun, unpublished], indicate that the hormone does not haphazardly "ping-pong" around the cell interior. Rather, the hormone enters the cell and is distributed to specific intracellular destinations by a series of protein-protein interactions which involve the hsp 70 family of intracellular vitamin D binding proteins.

For example, we now know from the work of Willnow et al. [Nykjaer et al., 1999; Christensen and Willnow, 1999] that vitamin D metabolites can enter target cells via internalized vesicles. The vitamin D metabolite stays bound to DBP, which is in turn bound by megalin and cubulin, members of the LDL superfamily of proteins. Once inside the cell there is interaction between the carboxy-terminal domain of megalin, which protrudes into the cytoplasm, and the amino-terminal domain of at least two different IDBPs, IDBP-1 and -3 [R. Chun, unpublished]. If one overexpresses either IDBP-1, the hsc-70 homolog, or IDBP-3, a mitochondrially-targeted hsp, and incubates transfected IDBP-overexpressing cells with fluorescently-labeled 25-hydroxylated vitamin D metabolite, one will observe a significant increase in the uptake of the labeled hormone. Moreover, if the protein–protein interaction is between megalin and IDBP-1 and the ligand is 1,25-$(OH)_2D_3$, then the ultimate

destination for that hormone and its chaperone is the unliganded VDR [J. Barsony and R. Chun, unpublished], residing in the perinuclear region of the cell. If, on the other hand, megalin interacts with IDBP-3, which contains an amino-terminal targeting sequence for the inner mitochondrial membrane, then the ultimate destination for the hormone is the mitochondria. Confirmation of a protein–protein interaction between a substrate-carrying IDBP molecule and a target enzyme, the 25-OHD-1-hydroxylase, has been accomplished with gst "pull-down" assays using the carboxy-terminal domain of the 25-OHD-1-hydroxylase as bait [R. Chun, unpublished]. Employing this substrate-accessible part of the enzyme to capture 25-OHD-1-hydroxylase-interacting proteins, we have shown that the grp-75-like IDBP-3, but not the hsc70-like IDBP-1, interacts with the 25-OHD-1-hydroxylase.

In summary, we propose that these hsp-mediated-chaperoning events are normally active in man but overly active in hormone-resistant New World primates. In New World primates, these chaperones function to compensate for the VDRE-BP-mediated vitamin D-resistant state by augmenting receptor function and increasing vitamin D hormone synthesis. We anticipate that further analysis of these events will more clearly define the protein-mediated vitamin D trafficking circuits that determine the fate of vitamin D metabolites in target cells harboring the VDR and/or vitamin D hydroxylases.

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