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DEVELOPMENTAL CHANGES IN RAT KIDNEY 1,25-DIHYDROXYVITAMIN D RECEPTOR[1]

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SUMMARY: Kidney 1,25-dihydroxyvitamin D receptor (VDR) was examined in both young and aged male Fischer 344 rats. Cytosols prepared by direct homogenization of the kidney indicated no significant difference in the amount of unoccupied VDR in young (149 ± 8 fmol/mg) and aged (155 ± 8 fmol/mg) rats. Binding of kidney VDR to DNA-cellulose, however, was significantly different for the two groups. The assay indicated that about 44% and 24% of the VDR prepared from young and aged rats, respectively, were bound to calf thymus DNA. Elution profiles from DNA-cellulose chromatography displayed the presence of two peaks from young kidneys, while a single broad peak was evident from aged rats. Immunoblot analysis confirmed the existence of two receptor bands at 52K and 50K. The presence of the 50K band was greatly diminished or absent in aged samples. The 50K receptor form was observed to elute from DNA-cellulose at a higher salt concentration than the 52K-form. Similarly, prepared receptor extracts from intestinal tissue produced only a single band at 52K. These results demonstrate for the first time that the rat kidney possesses two forms of the receptor which have different affinities for DNA.

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) is regarded as the hormonal form of vitamin D (1,2). The primary role of 1,25-$(OH)_2D_3$ has been characterized as the maintenance of calcium homeostasis. Typical of other steroid hormones, 1,25-$(OH)_2D_3$ interacts with an intracellular receptor protein (VDR) in target tissues (3-5). This complex is thought to bind to

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Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; VDR, vitamin D receptor.

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specific sequences of DNA and to result in the alteration of transcriptional events. One such event already characterized is the increased synthesis of an intracellular calcium-binding protein (6,7).

Age-related differences in steroid receptors have heightened research interest. Changes in either receptor number or the protein itself may have profound effects on the ability of an animal to respond to hormone. In this regard, weanling male rats possess a significantly higher number of specific binding sites for glucocorticoid receptor in their livers and pancreata than mature rats (8,9). Additionally, a recent report indicated that the ability of uterine estrogen receptors from aged mice to bind to DNA-cellulose was only one-half that of estrogen receptors in young mice (10).

A number of studies have shown that VDR is absent in embryonic tissues but appears at ~18 days of age in rats (11-13). Far less is known, however, about the characteristics of the receptor as an animal reaches maturity. Several reports have recently appeared detailing differences in VDR number and occupancy in both young and aged male rats (14-16). These studies described a marked decrease in receptor number in mature animals, in both intestinal and bone tissue, when measured against weanlings. We have examined, as part of an ongoing effort to study changes in VDR at different stages of development, whole kidneys in male Fischer 344 rats for receptor properties with regards to number, ability to bind to DNA, and sizing by SDS-PAGE.

MATERIALS AND METHODS

General: 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ were gifts from Dr. M. R. Uskokovic of Hoffmann-La Roche (Nutley, NJ). [^{3}H]-1,25-$(OH)_2D_3$ (90 Ci/mmol) was synthesized as previously described (17). The KTD buffer utilized throughout these studies contained 10 mM Tris-HCl, pH 7.4 (at 4°C), 5 mM dithiothreitol, and the indicated concentration of potassium chloride (i.e., KTD-500 contains 0.5M KCl). The TD buffer contained 10 mM Tris-HCl and 5 mM dithiothreitol, pH 7.4. Trayslol was purchased from Mobay Chemical Corp. (New York, NY). Soybean trypsin inhibitor was obtained from Sigma Chemical Corp. (St. Louis, MO). Hydroxyapatite was purchased from Bio-Rad Laboratories (Richmond, CA). DNA-cellulose was prepared according to the procedure of Litman (18) using calf thymus DNA (Sigma) and Whatman CF-11 cellulose (W & R Balston, Ltd., England). The 9A7 monoclonal antibody directed against VDR was a gift from Dr. J. W. Pike, Baylor College of Medicine (Houston, TX). Rabbit-anti-rat IgG (H+ L, mouse serum absorbed) was obtained from Zymed Laboratories (South San Francisco, CA). Proteins were electrophoretically transferred to Gene Screen hybridization membranes (NEN Research Products, Boston, MA). Electrophoresis grade reagents, Protein A gold colloid, and silver enhancement kit were obtained from Bio-Rad Laboratories. Protein was assayed by the method of Bradford (19). Concentrations of potassium were determined using a Perkin Elmer Model 5000 AA spectrophotometer at 404.7 nm.

Animals: Barrier-derived young (1 month) and aged (18 months) male Fischer 344 rats were obtained from the National Institute on Aging. Rats were maintained on a normal rat chow diet (Teklad 4%), and were allowed to acclimate to their environment for at least 4 to 6 weeks before use.

Receptor preparation: Kidneys were removed and placed in ice-cold, Tris-buffered saline containing Trayslol (50 KIU/ml). All subsequent steps were performed at 4°C. The kidneys were quartered and a 20% homogenate was prepared in KTD-500 containing 100 μg/ml soybean trypsin inhibitor (sbi) using three 10-s bursts of a Polytron tissue homogenizer, allowing 2 min between bursts. The homogenate was centrifuged at 230,000 for 30 min. The supernatant containing VDR was termed cytosol. Intestinal preparations were obtained by excising the proximal 20 cm of small intestine and flushing the lumen with ice-cold Tris-buffered saline with Trayslol. The mucosa was scraped free and a 20% homogenate prepared as described above.

Receptor assays: Unoccupied receptor concentrations were obtained by labeling samples of the cytosol in 24 nM [^{3}H]-1,25-$(OH)_2D_3$ ± a 200-fold excess of cold steroid at 4°C for 2 to 3 h with gentle agitation. A 4-fold excess of 24,25-$(OH)_2D_3$ was also added to all tubes to minimize interference from the vitamin D-binding protein. The samples were treated with 1% dextran-coated charcoal, and 50 μl aliquots of the receptor-hormone complexes were quantitated by hydroxyapatite in triplicate (20). The percentage of binding to DNA was determined as follows. After the charcoal treatment, 50 μl aliquots (triplicate) of the hormone-receptor complexes were incubated with 500 μl of a 50:50 slurry of DNA-cellulose in TD buffer in a final volume of 1 ml adjusting the [K^+] to approximately 150 mM. After 45 min with occasional gentle vortexing, the cellulose was pelleted and washed 2 X 1 ml TED buffer (1 mM EDTA) containing 0.5% Trition X-100. The pellets were then extracted 2 X 1.5 ml absolute EtOH, and the samples were dried and counted for radioactivity.

DNA-cellulose chromatography: Cytosol was labeled in 5 to 7 nM [^{3}H]-1,25-$(OH)_2D_3$ as described above. A 50:50 slurry of DNA-cellulose in TD buffer was added to the cytosol to yield an approximate [K^+] of 150 mM. After mixing intermittently for 1 h, the slurry was poured into a 1.5 cm diameter column. The column was washed at a flow rate of ca. 40 ml/h with 20 to 30 volumes of KTD-100 buffer, followed by a linear gradient from TD to KTED-500 (1 mM EDTA). Then 100 μl aliquots were removed from the collected fractions (ca. 3 ml/fraction) and counted for radioactivity to trace hormone-receptor binding. Samples of receptor-hormone complex were combined and precipitated with 40% saturated ammonium sulfate and analyzed in immunoblots.

Immunoblot analysis: Ammonium-sulfate-precipitated protein pellets were denatured directly in SDS-PAGE reducing buffer and resolved on 12% SDS-polyacrylamide mini-gels at 150 V according to the method of Laemmli (21). Proteins were electrophoretically transferred to Gene Screen hybridization membrane. Transfer was carried out at 100 V for 1 h by using a transfer buffer (0.025 M Tris-HCl, 0.19 M glycine) containing 0.1% SDS. The membrane was suspended for 1 h in phosphate-buffered saline (PBS, 10 mM sodium phosphate, pH 7.2, 0.9% NaCl) containing 10% Carnation Instant Milk (CIM). The membrane was then transferred to a solution of PBS/10% CIM containing a 1:2000 dilution of 9A7 monoclonal antibody and incubated overnight with agitation at 4°C. The membrane was washed for 30 min in PBS containing 0.3% Tween 20 with two changes of buffer. After a 1-h incubation in PBS/10% CIM, the membrane was suspended overnight at 4°C in a solution of PBS/10% CIM containing a 1:5000 dilution of rabbit-anti-rat antiserum. Following the previously described wash procedure, the membrane was incubated at 4 h in Protein A gold colloid. The membrane was washed as before, and the color enhancment of immunoblotted proteins was accomplished with silver lactate according to the manufacturer's instructions.

Statistics: The tabularized data from these experiments are reported as the mean ± SEM. Statistical analyses were performed by using Student's *t* test, and a confidence level of 95% or greater was considered significant.

RESULTS

Unoccupied kidney VDR status for young and aged Fischer rats are presented in Table I. There was no measurable difference in VDR concentrations between the two groups (149 versus 155 fmol/mg). VDR from aged rats, in contrast, displayed a marked decrease in binding to DNA-cellulose relative to VDR from young rats (24.1% versus 43.6%).

Representative elution profiles from DNA-cellulose chromatography of $1,25(OH)_2D_3$-VDR complexes prepared from the kidneys of young and aged Fischer rats are presented in Fig. 1. Salt elution revealed the presence of two VDR peaks in the chromatograms of VDR from young rats. Chromatography of kidney VDR from aged rats displayed only a single, broad peak using the same gradient elution conditions as for young rats.

VDR-containing fractions were pooled, precipitated with ammonium sulfate, and analyzed in Western blots (Fig. 2). Two protein bands of approximately equivalent intensity possessing molecular weights of 52K and 50K were evident in the material isolated from young rats (Lane A). The same two bands were visualized in the pooled samples from the aged preparations; however, the 52K-protein now predominates relative to the 50K-protein (Lane B). DNA-cellulose chromatography of intestinal preparations from either young or aged Fischer rats yielded a single sharp VDR peak in the elution profiles (data not shown). Immunoblot analysis from either intestinal VDR preparation resulted in the detection of a single protein band at 52K (Lanes C and D).

A more gradual salt gradient was employed to effect better separation of the two peaks present in the DNA-cellulose profile of VDR prepared from kidneys of young Fischer rats (Fig. 3). Fractions corresponding to the peak eluting at the lower salt concentrations (250 mM) were pooled and immunoblotted as before (Fig. 3 insert, Peak I). Similarly, the peak eluting at the higher salt concentration (300 mM) was analyzed by immunoblotting (Fig. 3 insert, Peak II). The material corresponding to Peak I was primarily composed of the 52K protein, while Peak II largely contained material associated with the 50K form. Unique low molecular weight bands of 21K and

TABLE I

Properties of kidney $1,25\text{-}(OH)_2D_3$ receptors prepared from young and aged Fischer 344 rats

	Young	Aged
Unoccupied[a]	149 ± 8 (8)[b]	155 ± 8 (8)
% DNA binding	43.6 ± 3.9 (8)	24.1 ± 2.3 (8)

[a]Expressed fmol/mg protein.
[b]The number in parentheses refers to the number of animals examined to determine the average value ± SEM.

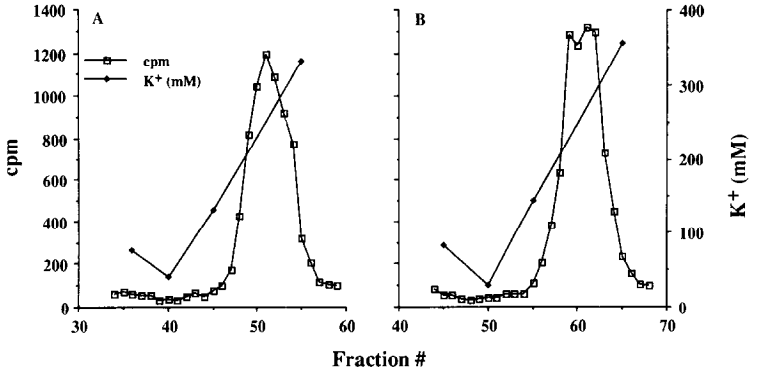

Fig. 1. Representative DNA-cellulose chromatography profiles of kidney VDR prepared from aged (A) and young (B) and aged Fischer 344 rats.

17K were also evident in the pooled fractions of the 52K and 50K peaks, respectively (Fig. 3, insert).

DISCUSSION

Examination of the kidney VDR in male Fischer 344 rats was conducted at two stages of development. There are evidently two forms of VDR present in similar amounts in cytosols prepared from rats 2 to 3 months of age. These two proteins exhibited distinctly different binding characteristics to DNA-cellulose, with the smaller, 50K protein, eluting at the higher salt

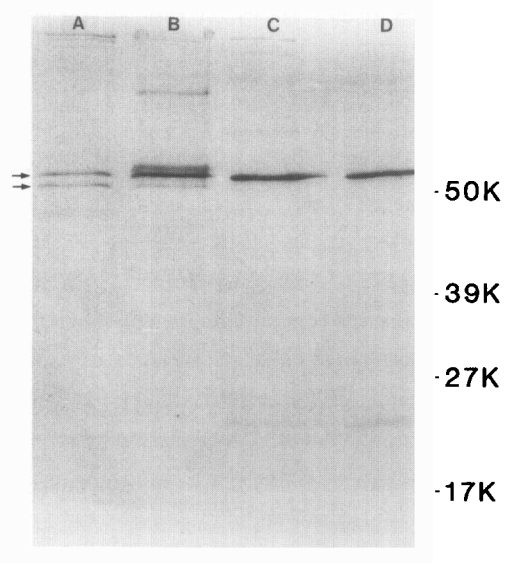

Fig. 2. Immunoblot analysis of VDR preparations from Fischer 344 rats following DNA-cellulose chromatography. Lane A, young kidney; lane B, aged kidney; lane C, young intestine; lane D, aged intestine. Kidney VDR appears as two protein bands at ca. 52K and 50K. Control blots (minus 9A7 monoclonal antibody) were used to determine nonspecific bands).

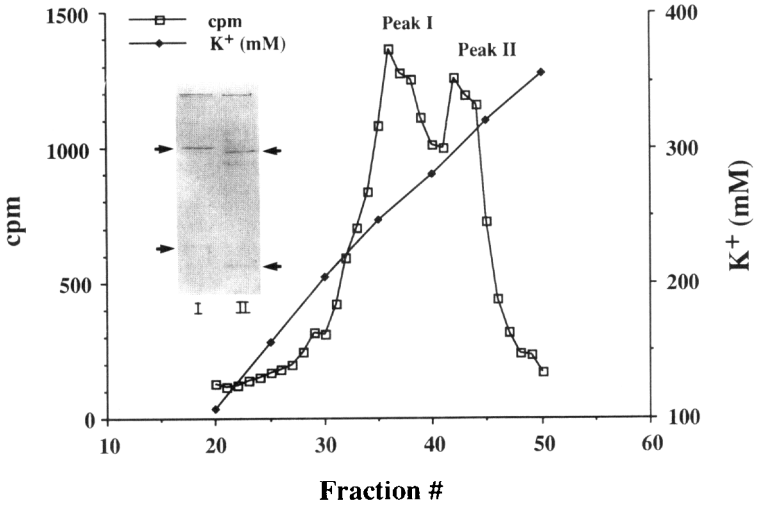

Fig. 3. Gradient elution of kidney VDR from DNA-cellulose prepared from young Fischer 344 rats. Fractions corresponding to each of the peaks were precipitated separately and blotted (insert). Peak I corresponds to the 52K protein and peak II corresponds to the 50K protein band.

concentration. Two forms of intestinal VDR also reportedly differing in binding behavior to DNA-cellulose have been observed in the avian system (22). It was proposed that in this circumstance, the smaller 58K protein was most likely derived from proteolysis of the larger 60K form.

Immunoblots (Fig. 3, insert) suggested that the two forms of VDR differ in their N-terminal region. Putative lower molecular weight proteolytic fragments presumably encompassing the respective DNA-binding domains and N-termini (23) displayed marked differences in molecular weight. The proteolytic fragments from the 52K and 50K forms of VDR were 21K and 17K, respectively. Differences in this region evidently have a profound effect on the binding characteristics to DNA, such that apparent truncation of the N-terminal results in tighter DNA binding.

Cytosols prepared from aged rats displayed little of the 50K protein, as evident in the DNA-cellulose chromatogram and concomitant immunoblot analysis. It is not clear from this work, however, if the 50K protein was present in the cytosols of the aged rats but was unable to bind to DNA. There was no difference in unoccupied kidney VDR concentrations between young and aged Fischer rats as measured by the hydroxyapatite assay, yet DNA binding was reduced by approximately one-half in the aged animals. This would be in agreement with expectations if the 50K protein were present but unable to bind to DNA.

Two-month-old Holtzman rats yielded similar results to those seen in the kidneys of young Fischer rats (data not shown), although the presence of the

50K protein band was attenuated. The rat kidney VDR, however, may not be representative of kidney VDR from other sources. Pig kidney VDR appears as a single 54K band as determined either by *in vitro* translation or metabolic labeling (24).

Alternatively, the two forms may represent phosphorylated and unphosphorylated VDR. Pike and Sleator (25) showed a reduction in electrophoretic mobility of phosphorylated VDR on SDS polyacrylamide gels resulting in an apparent increase in molecular weight from ~1 to 2K. A similar span in molecular weight (~2K) was observed between the two forms of kidney VDR in our experiments. The heavier 52K form may therefore be phosphorylated VDR. Although the role of VDR phosphorylation remains unclear, it is probably important in receptor action. If the 52K protein indeed represents phosphorylated receptor, then the higher quantities observed in aged rats may play a significant role in attenuating the biological response to 1,25-$(OH)_2$D, which is known to occur with aging (15,26).

In summary, two forms of VDR have been observed in immunoblots of preparations derived from young and aged Fischer rats. Approximately equal amounts of a 52K form normally associated with the rat's intestinal tissue and a unique, smaller 50K form were present in the kidneys of 2- to 3-month-old animals. In contrast, the aged (18-24 months) rats primarily exhibited the 52K receptor. Additional work necessary to delineate the source and physiological relevance of these two receptor forms is in progress.

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