Vitamin D Receptor Gene Expression in Mammalian Kidney

Lanting Liu, May Ng, Anthony M. Iacopino, S. Terence Dunn, Mark R. Hughes, and James E. Bourdeau

L. Liu, J.E. Bourdeau, Section of Nephrology, Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK
M. Ng, A.M. Iacopino, Department of Biomedical Sciences, Baylor College of Dentistry, Dallas, TX
S.T. Dunn, Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK
M.R. Hughes, Institute for Molecular Genetics, Baylor College of Medicine, Houston, TX
J.E. Bourdeau, Department of Physiology & Biophysics, University of Oklahoma Health Sciences Center, Oklahoma City, OK
J.E. Bourdeau, Nephrology Section, Medical Service, Department of Veterans Affairs Medical Center, Oklahoma City, OK

(Received January 10, 1994. Accepted May 2, 1994.)

ABSTRACT
The vitamin D-receptor protein and its mRNA were localized in microscope sections of paraffin-embedded mammalian kidneys by means of immunocytochemistry and in situ hybridization, respectively. A monoclonal antibody against chicken intestinal vitamin D receptor immunostained the nucleus and cytoplasm of cells within the distal convoluted tubule, connecting segment, and initial cortical collecting duct of both rats and pigs. Although fainter, immunostaining also was present over proximal tubular cells. (35S)UTP-labeled cRNA probes were detected over both the proximal and distal portions of the mouse nephron, but silver grain densities were 5.8-fold greater over the latter. In conclusion, localization of both the vitamin D-receptor protein and its mRNA in both the proximal and distal nephron of adult mammals suggests that the gene for this protein is expressed in cells at both of these sites. The intensity of immunostaining and the density of cRNA-associated silver grains suggest that vitamin D-receptor gene expression is greatest in the distal nephron.

Key Words: Calcitriol, distal convoluted tubule, connecting tubule, immunocytochemistry, initial cortical collecting duct, in situ hybridization, proximal tubule, vitamin D receptor mRNA

Calcitriol \( (1,25-(OH)_2\text{-vitamin D}_3 \) is synthesized by the proximal tubules and, in turn, acts on cells within both the proximal and distal nephron (1,2). The enzyme \( 25-(OH)\text{-vitamin D}_3 \ 1,25\)-hydroxylase, which is present in the mitochondria of proximal convoluted and straight tubules, catalyzes the formation of calcitriol (2,3). In both the convoluted and the straight portions of the proximal tubules, calcitriol increases the activity of \( 25-(OH)\text{-vitamin D}_3\text{-24-hydroxylase} \), the enzyme catalyzing the formation of \( 24,25-(OH)_2\text{-vitamin D}_3 \) (2,3). Calcitriol also induces the synthesis of both calbindin-D_28k and calbindin-D_9k in cells of the distal nephron, where calcitonin- and parathyroid hormone-stimulated transcellular calcium absorption occurs (4–7).

Calcitriol is a secosteroid hormone, the actions of which are mediated—at least in part—by an intracellular vitamin D-receptor protein (8). Cell fractionation studies suggest that the unoccupied receptor may exist in either the cytoplasm or the nucleus, but on occupancy, the receptor-calcitriol complex translocates to the nucleus (9–12). Therein, the occupied receptor interacts with a vitamin D-responsive element in the promoter region of target genes and exerts transcriptional control (12,13). The observations discussed above suggest that the vitamin D receptor might exist in cells of both the proximal and distal nephrons.

Previous studies suggest that the vitamin D receptor is present in cells of selective portions of the rat nephron. Thaw-mounted radioautography of kidney sections from vitamin D-deficient rats injected with \( {^3H}\text{calcitriol} \) revealed silver grains located primarily over the distal nephron, including the thick ascending limb of Henle's loop and the early and late portions of the distal convoluted tubule (14,15). \( {^3H}\text{calcitriol} \) uptake measurements in single nephron segments isolated by microdissection revealed specific binding in both proximal convoluted tubules and medullary thick ascending limbs (16). The purpose of this study was to investigate the location of the vitamin D receptor in cells of the mammalian nephron by the use of immunocytochemistry and in situ hybridization to identify the protein and its mRNA, respectively.

METHODS

Animals

This work was conducted in accord with the NIH Guide for the Care and Use of Laboratory Animals. For immunocyto-
chemistry, rats and pigs were used. Male Sprague-Dawley rats were obtained from Sasco, Inc. (Omaha, NE) and were maintained on a standard laboratory rodent diet (Rodent Blox®; Catalog Number 8604; Teklad, Inc., Madison, WI) and water ad libitum. They were anesthetized with ethyl ether, perfused intracardially (left ventricle) with chilled (4°C) normal saline for 5 to 10 min, and subsequently fixed by intracardiac perfusion with a chilled solution of 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS) for 20 min. The rats were killed by exsanguination from the right atrium during the fixation process. The kidneys were removed rapidly, and 3-mm-thick slices were cut perpendicular to the long axis. Farm swine were obtained from Sanquin Industries (Dallas, TX) and were maintained on a standard swine feed (Purina Hog Grower®;Ralston Purina Co., St. Louis, MO) and water ad libitum at the Caruth Research Laboratory of the Baylor University Medical Center (Dallas, TX). They were anesthetized with ketamine, 40 mg/kg body wt, and xylazine, 5 mg/kg body wt, im and were euthanized by intracardiac perfusion with a solution of 4% PFA and PBS. The kidneys were excised rapidly, and 5 × 5 × 2 mm³ tissue blocks were cut. For in situ hybridization, adult Swiss-Webster mice were obtained from the Zoology Department at the University of Oklahoma (Norman, OK) and were fed the same diet as the rats ad libitum. They were anesthetized with sodium pentobarbital, 60 mg/kg body wt, and their tissues were fixed by the same techniques described above for the rats.

**Tissue Processing**

For immunocytochemistry, the rat and pig kidney tissue samples fixed with PFA were immersed for 6 h in Bouin's solution. They were then washed for an additional 6 h in 50% ethanol with constant agitation and frequent changes of the washing solution. Tissues were stored in 70% ethanol for 24 h. Samples fixed with PFA were immersed for 6 h in Bouin's solution, washed for an additional 6 h in 50% ethanol, cleared in xylene, and mounted in Permount (Fisher Scientific, Pittsburgh, PA). In the final hydration steps were carried out at room temperature. First, the sections were deparaffinized with toluene and hydrated by successive immersion in aqueous solutions containing decreasing concentrations of ethanol. In the final hydration step, they were immersed in PBS. Endogenous peroxidase was eliminated by the incubation of the sections in 0.3% hydrogen peroxide in PBS for 30 min, followed by two washings with PBS. Normal rabbit serum (Elite ABC Kit; Vector Laboratories, Burlingame, CA) was applied to each section for 20 min as a blocking serum. Sections were then incubated with 10 µg/mL of the primary antibody for 48 h in a humidified chamber. In negative controls, the primary antibody was omitted. After three washings with PBS/0.01% Tween 20, sections were incubated with biotinylated rabbit antirat immunoglobulin G (Elite ABC Kit) for 1 h. After three additional washes, they were treated with biotinylated protein A (Vector Laboratories). 2.5 µg/mL, for 45 min. After three further washes, the sections were incubated with avidin-biotin horseradish peroxidase (Elite ABC Kit) for 1 h. The sections were then washed and incubated for 30 min with diaminobenzidine tetrahydrochloride-hydrogen peroxide-nickel chloride (Peroxidase Substrate Kit-DAB; Vector Laboratories). After a 10-min wash in PBS and a 5-min wash in water, some sections were counterstained with periodic acid–Schiff (PAS) reagent and/or hematoxylin, dehydrated in ethanol, cleared in xylene, and mounted in Permount for comparison of the values bracketing the asterisks either horizontally or vertically.

**TABLE 1. Silver grain densities over mouse kidney hybridized with (35S)UTP-labeled riboprobes for mouse vitamin D receptor**

<table>
<thead>
<tr>
<th>Structure</th>
<th>Antisense</th>
<th>Sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomeruli</td>
<td>0.145 ± 0.012</td>
<td>0.110 ± 0.006</td>
</tr>
<tr>
<td>(N = 12)</td>
<td>(N = 14)</td>
<td></td>
</tr>
<tr>
<td>Proximal Tubules</td>
<td>0.240 ± 0.010</td>
<td>0.169 ± 0.011</td>
</tr>
<tr>
<td>(N = 25)</td>
<td>(N = 20)</td>
<td></td>
</tr>
<tr>
<td>Distal Tubules</td>
<td>0.603 ± 0.028</td>
<td>0.191 ± 0.018</td>
</tr>
<tr>
<td>(N = 16)</td>
<td>(N = 18)</td>
<td></td>
</tr>
<tr>
<td>Collecting Ducts</td>
<td>0.134 ± 0.008</td>
<td>0.158 ± 0.010</td>
</tr>
<tr>
<td>(N = 10)</td>
<td>(N = 22)</td>
<td></td>
</tr>
</tbody>
</table>

*Values are means ± SE. Mean values for antisense and sense riboprobes over emulsion-coated glass were 0.022 and 0.014 grains/µm², respectively. These have been subtracted from the grain densities reported for the structures in the table. Grain densities (in grains per square micrometer) over emulsion-coated tissue sections not exposed to radioactively labeled riboprobes were 0.023 ± 0.013 (N = 14) for glomeruli, 0.022 ± 0.001 (N = 15) for proximal tubules, and 0.023 ± 0.003 (N = 17) for distal tubules. Six mice were studied. N, number of structures analyzed. NS, not significant. * P < 0.05.
from the values appearing in Table 1. Results are presented as the mean ± SE. Statistical inferences were derived from one-way analysis of variance (24) in conjunction with the Bonferroni t test for the comparison of multiple means (25) or the t statistic for two means (26). P values less than 0.05 were considered significant and are indicated by an asterisk.

RESULTS

Immunolocalization of the vitamin D receptor in the kidneys of rats and pigs was similar. In noncounterstained sections of rat kidney, the cytoplasm and nuclei of the majority of cells in the distal convoluted and the connecting tubules stained brown with the avidin-biotin procedure, whereas staining was absent over these same structures in immunocontrol sections (Figure 1). In counterstained sections, the vitamin D receptor was observed primarily over cells within the distal nephron—including the distal convoluted tubule, the connecting tubule, and the initial portion of the cortical collecting duct—in both species. Faint immunostaining was also present over the cytoplasm of proximal convoluted tubule cells that had

Figure 1. Noncounterstained rat kidney cortex. (A) Immunostaining for the vitamin D receptor demonstrating that both nuclei (arrowheads) and cytoplasm of cells within tubules located in arcades (a) stain darkly. Immunostaining was fainter in proximal tubular cells (p). (B) Negative immunocontrol. Scale bars, 50 µm.
been counterstained with PAS/hematoxylin (Figure 2). Vitamin D receptor staining was absent from cells in the renal corpuscle, Henle’s loop, and medullary collecting ducts.

In situ hybridization experiments to localize the mRNA for the vitamin D receptor in adult mouse kidneys revealed silver grains that were concentrated predominantly over cells of the distal nephron, with fewer grains over proximal tubules (Figure 3). Glomeruli and cortical collecting ducts beyond the initial portion did not reveal significant labeling. These results are summarized in Table 1.

**DISCUSSION**

In this study, the vitamin D receptor was localized primarily in cells of the distal nephron, including the distal convoluted tubule, the connecting tubule, and the initial portion of the cortical collecting duct. These findings are concordant with those of previous studies that identified the vitamin D receptor in the distal nephron by thaw-mount radioautography (14,15), 

\[ \text{[3H]} \text{calcitriol uptake (16), or immunocytochemistry (27). Likewise, the mRNA for the vitamin D receptor was most abundant in cells of the distal nephron.} \]

Although the vitamin D-receptor protein and its mRNA localized most prominently to the distal nephron, they were also detectable in proximal tubules. In this regard, our findings are in agreement with the weak localization of \( \text{[3H]} \text{calcitriol to the nuclei of proximal tubules in vitamin D-deficient Sprague-Dawley rats (15), with [3H]calcitriol binding to isolated proximal tubules microdissected from vitamin D-deficient rats (16), and with the immunocytochemical localization of the vitamin D receptor to the proximal tubules of human kidneys (27).}\]

To our knowledge, this study is the first to localize the mRNA for the vitamin D receptor within specific nephron segments. Hybridization with antisense RNA probes was significantly greater than that with sense RNA probes in both the proximal and the distal nephrons. By subtracting the sense from the antisense values, it is possible to estimate the specific binding over cells in each nephron segment. For the proximal tubules, this value is 0.071 (i.e., 0.240 - 0.169) grains/\( \mu m^2 \). For the distal tubule, the value is 0.412 (i.e., 0.603 - 0.191) grains/\( \mu m^2 \). Thus, the specific binding of \( [35S]UTP-cRNA \text{ to distal tubular cells was 5.8-fold greater than that observed to cells of the proximal nephron. These findings are concordant with the differences in immunostaining between cells of the proximal tubules and distal nephron discussed above.}\]

In this study, the vitamin D-receptor protein and its mRNA were localized in kidney tissue sections by immunocytochemistry and in situ hybridization, respectively. These procedures were performed in different species for practical reasons. We chose the pig and the rat for immunocytochemistry, because the monoclonal antibody directed against the chicken intestinal vitamin D-receptor protein cross-reacts with vitamin D receptors in kidney tissues from these species (19,20). Localization of the mRNA for the vitamin D receptor by in situ hybridization was performed in mouse kidney sections because the murine vitamin D-receptor cDNA, which served as the starting point for the synthesis of the \( [35S]UTP-labeled riboprobes, was cloned in one of our laboratories (M.R.H.) and, therefore, was readily available. Because we localized the vitamin D-receptor protein in renal tubular cells of the pig and rat, but localized its mRNA in renal tubular cells of the mouse, we cannot, strictly speaking, conclude that the vitamin D-receptor protein and its mRNA have been colocalized in the same cells. However, these findings, in conjunction with the high degree of homology of nephron structure between mammalian species (28), suggest that the gene for the vitamin D receptor is transcribed and its message is translated within both the proximal and distal portions of the nephron.

On the basis of the results of cell fractionation experiments performed in nonrenal tissues, there is controversy regarding the subcellular distribution of the unbound vitamin D receptor (13). The calcitriol-bound receptor is present in the nucleus, and the unbound receptor may be present within both the cytoplasm and the nucleus (13). In our study, we used a monoclonal antibody that recognizes both the occupied and the unoccupied forms of the receptor and observed immunostaining in both the nucleus and the cytoplasm. Because hematoxylin obscured immunostaining within the nuclei, we examined sections that were not counterstained. They demonstrated both nuclear and cytoplasmic staining. For optimal identification of the nephron segments, we counterstained some sections with PAS/hematoxylin or hematoxylin alone, which did not obscure cytoplasmic immunostaining. From our experiments, we suggest that the vitamin D receptor is present in both the nuclei and the cytoplasm of proximal and distal tubular cells, but we cannot differentiate between the bound and unbound forms.
Figure 3. Bright-field (left) and dark-field (right) photomicrographs of mouse kidney cortex sections hybridized with \(^{35}\text{S}\)UTP-labeled cRNA for vitamin D-receptor mRNA (A to D) or with \(^{35}\text{S}\)UTP-labeled sense RNA (E and F). Arrowheads, distal nephrons; g, glomerulus; bv, blood vessel. Hematoxylin and eosin counterstain. Scale bars, 50 \(\mu\)m.
Our study fails to provide evidence for vitamin D–receptor gene expression in the glomerulus. Vitamin D–receptor protein was undetectable by immunocytochemistry, and hybridization of vitamin D–receptor mRNA was not significantly greater with antisense than with sense riboprobes. Our study is not necessarily inconsistent with thaw-mount radioautography showing the concentration of [3H]calcitriol-associated silver grains over glomeruli in vitamin D–deficient rats (14, 15). Calcitriol is bound to a 58-kd vitamin D–binding protein (29), the size of which predicts that it should be filtered at the glomeruli. Accordingly, the findings discussed above (14, 15) may reflect the uptake of the [3H]calcitriol/vitamin D–binding protein complex by the podocytes. This hypothesis is supported by the observation that the vitamin D–binding protein is sequestered in the kidneys of both rabbits and vitamin D-deficient rats (32), and this approach, when applied to the detection of the renal tubule. Accordingly, the presence or absence of vitamin D receptors and the transcription of the vitamin D–receptor gene in cells of the glomeruli remain uncertain.

Unexpectedly, this study failed to detect vitamin D receptor mRNA or protein over the thick ascending limb of Henle’s loop. There are several lines of evidence that suggest that the vitamin D receptor might be present in this portion of the nephron. First, thaw-mount radioautography revealed [3H]calcitriol binding to cells of the thick ascending limbs of Henle’s loop in vitamin D–deficient rats (15). Second, single medullary thick ascending limbs isolated from the kidneys of vitamin D–deficient rats and incubated with [3H]calcitriol showed specific uptake (16). Third, calbindin-D9k is present in the thick ascending limb of Henle’s loop in the rat (6). These observations question the sensitivity of the immunocytochemical and in situ hybridization methods in detecting the vitamin D–receptor protein and its mRNA, respectively, in all portions of the renal tubule. Accordingly, the presence or absence of vitamin D–receptor gene expression in this segment of the nephron requires further investigation.

We have recently developed reverse transcription–polymerase chain reaction techniques to detect calbindin-D28k mRNA in singly dissected nephron segments (32), and this approach, when applied to the detection of vitamin D–receptor mRNA, should be useful in dealing with this issue.

In conclusion, the vitamin D–receptor protein and its mRNA are present in both the proximal and the distal nephron of the mammalian kidney, suggesting that the gene for the receptor is expressed in the cells of both tubular segments. As assessed by immunocytochemical staining of the protein and quantitative in situ hybridization of the mRNA, gene expression is greatest in the cells of the distal nephron.

ACKNOWLEDGMENTS

We gratefully acknowledge the resources of the Molecular Pathology Core Laboratory of the Oklahoma Center for Molecular Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK, and the staff of the Samuel Lloyd Noble Electron Microscopy Laboratory, University of Oklahoma, Norman, OK. We thank Dr. Bao Le Wang of the University of Oklahoma Health Sciences Center for the rat kidneys. This work was supported in part by Grants DK35985 (J.E. Bourdeau) and DK14469 (M.R. Haussler) from the National Institute of Diabetes and Digestive and Kidney Diseases and by intramural funding from the Baylor College of Dentistry (A.M. Iacopino).

REFERENCES


