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NUCLEAR TESTICULAR 1,25-DIHYDROXYVITAMIN D_3 RECEPTORS IN SERTOLI CELLS AND SEMINIFEROUS TUBULES OF ADULT RODENTS

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Summary: $1,25(OH)_2D_3$ receptors were studied in whole testes, Sertoli cells, seminiferous tubules, Leydig cells and spermatogonia of adult NMRI mice and SD rats. Specific reversible high affinity binding (K_D 1.4 x 10^{-10}M; N_{max} 72 fmol/mg protein) by a 3.5 S macromolecule was demonstrated in whole testes, Sertoli cells and seminiferous tubules. With identical techniques, no receptors were found in Leydig cells despite previous reports of $1,25(OH)_2D_3$ actions on Leydig cell function.

In a previous study we demonstrated 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) receptors in rat testis (1, 2). Recently we (3, 4) and others (5, 6) postulated that cellular $1,25(OH)_2D_3$ receptor status is related to cell proliferation and differentiation. This consideration led us to reexamine cellular distribution of $1,25(OH)_2D_3$ receptors in testis, specifically Leydig cells vs. seminiferous tubules.

Material and Methods:

Chemicals: 1,25-dihydroxy [26,27-methyl-^{3}H] cholecalciferol (158 Ci/mmol), 25-hydroxy [23,24-^{3}H] cholecalciferol (85 Ci/mmol) were obtained from Amersham/Buchler Co. (Braunschweig). Radiochemical purity by HPLC 93-98% with no other D metabolites demonstrated. Radioinert chromatographically pure $1,25(OH)_2D_3$, $25(OH)D_3$ and 24 R, $25(OH)_2D_3$ from Duphar Co. (Amsterdam). Bovine serum albumin [^{14}C] methylated (20 µCi/mg protein); ovalbumin [^{14}C] methylated (20 µCi/mg protein), y-globulin [^{14}C] methylated (20 µCi/mg protein), [^{125}I] cAMP (150 Ci/mmol) from NEN (Dreieichenhain). Hydroxylapatite, dithiothreitol, Triton X-100, Na-molybdate from Sigma (Munich), colloidal PVP (polyvinylpyrolidone) coated silica Percoll^R from Pharmacia Fine Chemicals AB (Uppsala), Hepes-buffered minimum essential medium (M 199), FCS, collagenase (129 U/mg) by Seromed Biochem KG (Berlin), HCG (5000 U/mg, Primogynol^R, Schering (Berlin), NAD, dehydroepiandosteron, nitrobluetetrazoliumchloride (NBT) Serva (Heidelberg), FSH (75 IU/mg) Serono (Freiburg).

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Animals:

Non-rachitic adult SD rats (8 weeks, 250 g) and NMRI mice (7 weeks, 40 g) were raised on a standard diet. Testes and intestinal mucosa (as control for methodology) were immediately removed after cervical dislocation.

Cell preparation (7,8): Leydig cells were prepared from decapsulated testes which were placed in Hepes buffer MEM (M 199) (0.2 M Hepes, 0.1% BSA, 0.01% penicillin, 0.02% streptomycin; pH 7.4; 4°C; 95% O_2, 5% CO_2) including 1 mg collagenase (129 U) in 5 ml buffer. After 30 min incubation (gentle shaking 60 osc/min; 34°C; 95% O_2; 5% CO_2) tubular fragments were sedimented (5 min, room temperature). The supernatant containing 20-40% crude Leydig cells was layered on a 0-90% linear continuous gradient of Percoll in M 199 (7); centrifugation for 20 min at 800 g. Band 3 (7) contained 95% Leydig cells with a yield of 6-9 x 10^5 cells/mouse. Identification by NBT and cell viability by trypan blue exclusion (fin.conc. 0.01%) (>95% of cells). 8-fold cAMP stimulation by HCG or LH (25 IU/ml) but not FSH (10 IU/ml) (8).

Seminiferous tubules were prepared from the above sediment which was washed 3x in M 199 and layered on a 0-90% Percoll density gradient. Trypan-blue exclusion, NBT histochemistry (<5% positive) and light microscopy as above. No cAMP stimulation by HCG.

Sertoli cell preparation after Dorrington (9). The above tubular fragments were incubated (30 min, 34°C, 95% O_2, 5% CO_2; 10 ml M 199) with constant shaking (60 osc/min) using 10 µg collagenase. Material was filtered through polyester gauze (200 µm) allowing spermatogonia and Sertoli cells to pass. After centrifugation (2 min, 800 g) the pellet (Sertoli cells) was washed (3 x; M 199). Viability and purity of preparation as indicated above: 7-fold stimulation of cAMP with FSH, but not HCG or LH; no NBT indicator reaction).

Spermatogonia: (germinal cell-enriched fraction) according to Tung (10) from the above filtrate after removal of the (Sertoli cell) pellet. The supernatant, after centrifugation (800 g; 5 min) and resuspension (M 199) was layered onto a gradient containing 2% BSA. After sedimentation in BSA for 10 min, most of the 2% BSA fraction was removed and the rest (1 ml) resuspended in M 199. Viability and purity of germ cell fraction examined as above. Negative for NBT indicator, no cAMP stimulation after HCG, 5-fold stimulation with FSH.

Cytosol preparation: As described previously (1-4), the freshly prepared cell suspension were sonicated (15-s bursts, Sonofier cell disruptor). After suspension of cells in four volumes (w/v) of KCl containing hypertonic buffer,material was sonicated and centrifuged at 500 g, 10 min, 4°C to yield a crude supernatant fraction. The buffer (KTEDMo) contained: 0.4 M KCl/HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 10 mM Na-molybdate, pH 7.4, 4°C). A purified cytosol fraction was prepared by centrifugation at 105,000 g for 60 min at 4°C (Beckman Instruments Ultracentrifuge). The cytosol was meticulously kept at 4°C throughout. Cytosol protein after Lowry (11).

Nuclear preparation: Method as described previously (12). In brief, material was homogenized in 4 volumes (w/v) of KCl-free buffer (TED) (10 mM Tris HCl, 1.5 mM EDTA, 1 mM DTT, 10 mM sodium molybdate; pH 7.4; 4°C) using a Polytron homogenizer. Homogenate was centrifuged (5000 g; 10 min; 4°C) to yield the nuclear pellet. A crude chromatin fraction was prepared by resuspending the pellet 3 x in the above buffer with 0.5% Triton X-100 (pH 7.4) followed by centrifugation (10,000 g; 10 min). The resulting crude chromatin pellet was then extracted with 0.4 M KCl-TED for 45 min with frequent mixing. Chromatin extract was centrifuged (5,000 g/

10 min) and resulting supernatant subsequently centrifuged at 100,000 g for 1 h.

Sucrose density gradient analysis and hydroxylapatite assay as described in detail previously (1, 4, 12): In brief, linear 5-20% sucrose density gradient in KTEDMo-buffer (4 ml) was made using a self designed gradient former. Cytosol or nuclear samples (0.2 ml) were incubated with 1 nM [^{3}H] 1,25(OH)$_2$D$_3$ alone or in addition with 100-fold molar excess of 1,25(OH)$_2$D$_3$; 25(OH)D$_3$; 1-α(OH)D$_3$; 24R, 25(OH)$_2$D$_3$. They were then carefully layered on top of pre-equilibrated (2 h, 4°C) gradients and centrifuged (255,000 g; 21 h; 4°C, SW-60 rotor Beckman Instruments Co). 7-drop fractions were collected. The sedimentation rate (in Svedberg units, S) for proteins was calculated using [^{14}C] labeled ovalbumin (3.7 S) or y-globulin (7.3 S).

Saturation analysis according to Scatchard (13). Aliquots of diluted cytosol (0.1 ml/0.6 mg protein) or nuclear extracts (0.1 ml/0.3 mg protein) were incubated with varying concentrations (0.1-10 nM) of [^{3}H] 1,25(OH)$_2$D$_3$ in the absence (total binding) or presence (non-specific binding) of a 100-fold molar excess of unlabeled 1,25(OH)$_2$D$_3$ for 16 h at 4°C. Bound [^{3}H] 1,25(OH)$_2$D$_3$ was determined using the above hydroxylapatite assay.

Binding of 1,25(OH)$_2$D$_3$ receptors to DNA cellulose. DNA cellulose (0.3 mg DNA/ml) according to Alberts and Herrick (14) using highly polymerized calf thymus DNA (type I, Sigma^R) and Whatman CF-11 cellulose. Each column was equilibrated in TED for 16 h at 4°C prior to use and run at a flow rate of 5 ml/h/cm^2. Samples were loaded onto the columns, washed with 3 volumes of TED and eluted in 10 volumes with a linear gradient from 0.1 M to 0.6 M KCl in TED. Nuclear extracts (0.6 ml/1.8 mg protein) of cell fractions were incubated for 2 h at 4°C with [^{3}H] 1,25(OH)$_2$D$_3$ prior to chromatography on DNA cellulose.

Results:

1,25(OH)$_2$D$_3$ binding in cytosol and nuclear fractions of whole testis and its various cellular components

In whole rat testis we confirmed our previous finding of high affinity binding (1, 2) and similar binding was also noted in NMRI mouse whole testes (N_{max} = 85 fmol/mg protein; K_D 1.4 x 10^{-10}M). In Leydig cell preparations, no significant binding could be detected in an assay which reproducibly measured 5 fmol/mg protein specific binding of 1,25(OH)$_2$D$_3$ in intestinal mucosa preparations (3). In seminiferous tubules specific binding of 1,25(OH)$_2$D$_3$ could be demonstrated both in the cytosol and in the nuclear fraction (K_D 1.4 x 10^{-10}M; N_{max} 80 fmol/mg protein) with a single class of non-interacting binding sites. Binding was reversible (i.e. abolished by 100-fold molar excess of 1,25(OH)$_2$D$_3$), specific (not abolished

by 100-fold molar excess of 25(OH)D_3; 24R,25(OH)$_2D_3$ or 1α(OH)D_3) and was mediated by a macromolecule migrating at approximately 3.5 S.

Analyses of Sertoli cell and spermatogonia fractions respectively showed specific binding in Sertoli cells (72 fmol/mg protein; see fig. 1 C and D) and no binding in spermatogonia.

Binding of [^{3}H] 1,25(OH)$_2D_3$ receptors to DNA cellulose

As shown in fig. 1B, 1,25(OH)$_2D_3$ nuclear receptor complex of seminiferous tubules associated with DNA under conditions of low ionic strength (0.1 M KCl) and could be eluted as single peak at 0.25 M KCl. Incubation of the nuclear fraction with 100-fold molar excess of 1,25(OH)$_2D_3$ completely obliterated the radioactive peak on the DNA cellulose chromatogram, demonstrating the specific hormone binding character of the receptor.

Discussion:

The present results complement our previous studies documenting 1,25(OH)$_2D_3$ receptors in, and functional actions of 1,25(OH)$_2D_3$ on, whole adult rat testes (1, 2). Typical 1,25(OH)$_2D_3$ receptors (15-17) were localized in both cytosolic and nuclear fractions of seminiferous tubules and Sertoli cells. The sedimentation coefficient (3.5 S) corresponds to the one concomitantly found in intestinal mucosa (data not shown) and could be easily differentiated from the 4 S serum binding protein and the 6 S (25(OH)D_3 tissue binding protein (18). Scatchard analyses revealed presence of a single class of non-interacting binding sites. Binding sites exhibited high binding selectivity (1,25(OH)$_2D_3$ > 25(OH)D_3 > 24R, 25(OH)$_2D_3$), low binding capacity (N_{max} = 72 fmol/mg protein of Sertoli cells) and high binding affinity, i.e. low equilibrium dissociation constant (K_D = 1.4 x 10^{-10}M). Circulating 1,25(OH)$_2D_3$ levels in rodents are in the range of such K_D (19). Partial receptor occupancy in vivo is suggested by the finding that 1,25(OH)$_2D_3$ receptor recovery was twice as high in cytosolic fractions of testes prepared at high (KTED) as opposed to low ionic strength (TED). Previous obser-

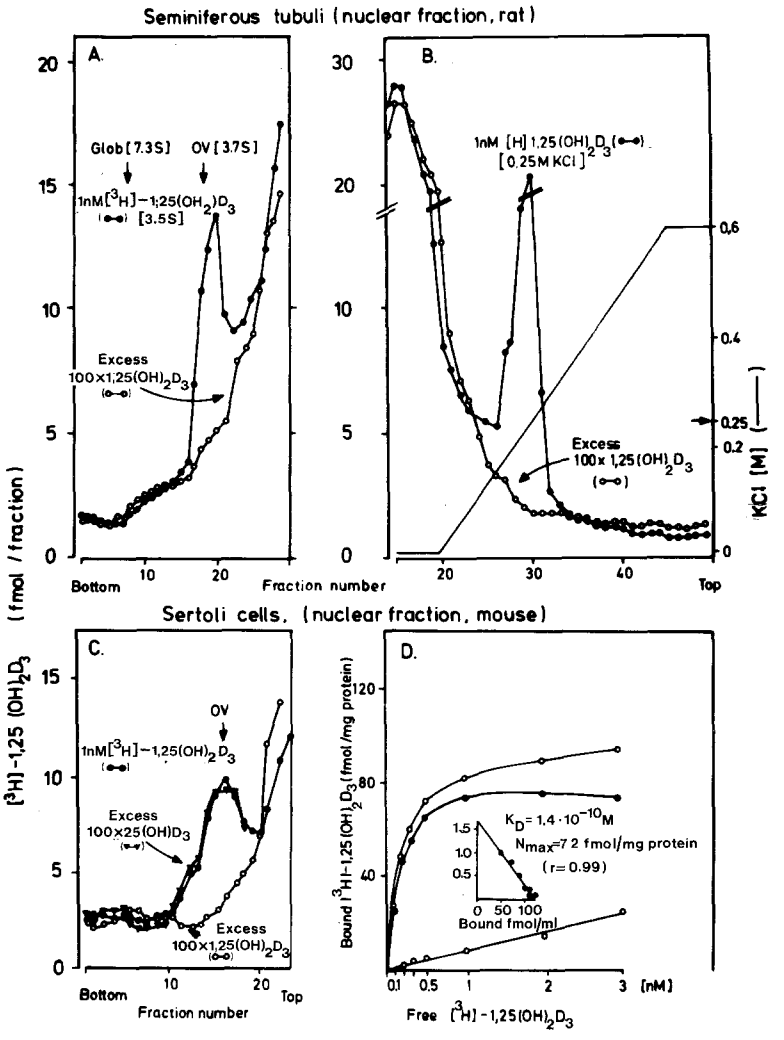

Fig. 1:

[^{3}H] 1,25(OH)$_2$D$_3$ receptor binding in Sertoli cells and seminiferous tubules of rodents (rats, mice).

Panel A: Sucrose density (5-20%) gradient analyses (21 h, 4°C, 255,000) of [^{3}H] 1,25(OH)$_2$D$_3$ binding in the absence (●——●) and presence of 100-fold molar excess of 1,25(OH)$_2$D$_3$ (○——○).

Panel B: Elution of specific 1,25(OH)$_2$D$_3$ receptors from DNA cellulose columns. Nuclear fraction of seminiferous tubules (0.6 ml/1.8 mg protein) incubated with 2 nM [^{3}H] 1,25(OH)$_2$D$_3$ in the absence (●——●) and presence (○——○) of 100-fold molar excess of 1,25(OH)$_2$D$_3$. Subsequently layered on 1.5 x 6 cm DNA cellulose column. Elution using linear KCl gradient.

Panel C: Sucrose density gradient analyses of [^{3}H] 1,25(OH)$_2$D$_3$ binding to nuclear fraction of mouse Sertoli cells analogous to rat seminiferous tubules (Panel A).

Panel D: Saturation analyses of [^{3}H] 1,25(OH)$_2$D$_3$ in nuclear fraction of Sertoli cells (mouse). Nuclear fractions (0.2 ml/0.6 mg protein) were incubated with increasing concentrations (0.1-3 nM) of [^{3}H] 1,25(OH)$_2$D$_3$ (16 h, 4°C) in the presence and absence of 100-fold molar excess of non-radioactive 1,25(OH)$_2$D$_3$. Scatchard analyses yielded a straight regression curve (K_D 1.4 x 10^{-10}M; N_{max} 72 pmol/mg protein).

vations (17) demonstrated that 90% of the unoccupied receptor of $1,25(OH)_2D_3$ remain in the nuclear fraction when chick intestinal mucosa is homogenized in low salt buffer, but appear in the cytosolic fraction when homogenates are prepared in high salt buffer, suggesting translocation of unoccupied nuclear receptors into the cytosolic compartment at high ionic strength.

Within the detection limits of the method (5 fmol/mg protein) in intestinal mucosa; 3), $1,25(OH)_2D_3$ receptors were not found in all testicular structures: they were present in seminiferous tubuli and Sertoli cells, but not in Leydig cells or spermatogonia. Absence of receptors on Leydig cells is particularly surprising as earlier studies documented $1,25(OH)_2D_3$ actions on HCG (LH) stimulated cAMP generation of rat testes in vivo (2). We cannot exclude that such $1,25(OH)_2D_3$ actions are mediated by non-genomic, e.g. liponomic mechanisms.

The role of $1,25(OH)_2D_3$ on testicular (or ovarian; 21) function is unknown. Calcium binding protein, a $1,25(OH)_2D_3$-dependent gene product, is expressed in testes (20). $1,25(OH)_2D_3$ receptors have recently been demonstrated on many organs unrelated to calcium metabolism. On the basis of studies in dermal cells (4) or blood cell lines (3), both we (3, 4) and others (5,6) concluded that $1,25(OH)_2D_3$ influences cell proliferation and differentiation. It is therefore of particular interest that (within the sensitivity of the method) $1,25(OH)_2D_3$ receptors were not demonstrable on spermatogonia. This is also surprising, since in rodents whole testes $1,25(OH)_2D_3$ receptor concentration was shown to increase during puberty in parallel with spermatogenesis (22). Further studies must clarify whether possible actions of $1,25(OH)_2D_3$ on spermatogenesis are mediated indirectly via $1,25(OH)_2D_3$ receptor bearing Sertoli cells.

The chance finding of cAMP stimulation by FSH in spermatogonia, but not Sertoli cells, which was obtained when we tried to further characterize the preparations used, merits further investigation.

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