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A ROLE FOR THE CYTOSKELETON IN RENAL VITAMIN D METABOLISM

Jonathan L. Taft and Richard G. Larkins

Department of Medicine, Royal Melbourne Hospital,
Parkville, Victoria, Australia

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Conversion of circulating 25-hydroxyvitamin D_3 ($25(OH)D_3$) to its active metabolite 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) occurs in the renal tubule mitochondrion. Recent reports have implicated the cytoskeleton in certain other steroid metabolizing cells as a mediator of a rate-limiting mitochondrial transport step. Whilst the activity of the renal converting enzyme, a typical steroid hydroxylase, is known to be regulated closely by a number of well studied factors, no information is available to indicate whether an analogous transport step is relevant to the regulation of vitamin D metabolism. Cytochalasin B and vinblastine were used as chemical antagonists of the microfilamentous and microtubular elements of the cytoskeleton. Both agents inhibited the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by isolated vitamin D-deficient chick renal tubules in a dose-dependent manner. At the concentrations required to inhibit $25(OH)D_3$-1α-hydroxylase activity in whole cells, these agents inhibited neither isolated mitochondrial $1,25(OH)_2D_3$ production, nor $24,25(OH)_2D_3$ synthesis by vitamin D-replete tubules. The cytoskeletal antagonists were found to increase the content of labelled $1,25(OH)_2D_3$ and $25(OH)D_3$ in a mitochondrial fraction prepared by Percoll fractitionation of tubule cells pre-exposed to the antagonists and labelled $25(OH)D_3$ substrate. The data suggest that disruption of the cytoskeleton may result in inhibition of transport of newly synthesised $1,25(OH)_2D_3$ out of the mitochondion and through the cell, and accumulating $1,25(OH)_2D_3$ may oppose its further synthesis. This is consistent with a transport process mediated by the cytoskeleton being involved in the regulation of renal vitamin D metabolism.

The active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), maintains calcium and phosphate homeostasis by its steroid-type actions on its main target organs; bone, gut and kidney. The $25-(OH)D_3$-1α-hydroxylase enzyme, a typical steroid hydroxylase (1), located in the renal tubules cell mitochondrion, produces $1,25(OH)_2D_3$ from circulating 25-hydroxyvitamin D_3 ($25(OH)D_3$). In vitamin D-replete states, production of the alternative metabolite $24,25(OH)_2D_3$ is favoured. The tight regulation of activity of 1α-hydroxylase has been extensively studied (2); however little is known about vitamin D metabolite transport within the cell, and its importance in $1,25(OH)_2D_3$ production.

Some reports have implicated the cytoskeleton as a mediator of a mitochondrial transport step for steroids that is rate-limiting for their further metabolism by steroid hydroxylases, in adrenal and testicular cells (3-12). Because of these observations we have investigated whether the cytoskeleton plays

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a role in the regulation of intracellular transport of vitamin D metabolites and thus affects renal vitamin D metabolism. The cytoskeleton, consisting of microtubular and microfilamentous networks, is involved in cell motility, division and intracellular transport, particularly of secretory products (13,14). Data obtained by the use of cytochalasin B and vinblastine as a microfilament and microtubule antagonist respectively, are presented which supports the concept of an involvement of the cytoskeleton in the intracellular transport of vitamin D metabolites and in vitamin D metabolism.

MATERIALS AND METHODS

Renal Tubule Preparation

One day old white Leghorn-Australorp cross cockerels were raised on a vitamin D deficient diet based on soybean meal, containing 1% added calcium and phosphorus, for 3-4 weeks before sacrifice. Non-vitamin D-deficient chicks were raised on a standard commercial diet. Kidneys were rapidly removed, dissected and rinsed in ice-cold buffer of 120 mM NaCl, 4 mM KCl, 2mM NaH_2PO_4, 1.6 mM $MgSO_4$, 1.25 mM $CaCl_2$, 10 mM glucose, 0.1% gelatin, 10 mM Hepes/NaOH pH 7.4. An isolated tubule suspension was prepared by collagenase digestion (4mg/gm) of the kidneys for 20 min at 37°C, followed by washing of the digests and filtration through nylon mesh as previously described (15).

Tubule $1,25(OH)_2D_3$ production

Identical 1.5 ml aliquots of tubule suspension were preincubated for 20 min with the agent under study at 37°C in a shaking water bath, then incubated for a further 20 min with 25-hydroxy[26(27)-methyl-^{3}H] vitamin D_3 substrate at 50 nM and 0.05 µCi/vial specific activity. Vitamin D metabolites were extracted by methanol:chloroform (2:1 v/v) by the method of Bligh and Dyer(16) and separated by normal phase HPLC (15). The $25(OH)D_3$ and $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ peaks were identified by co-elution with authentic standards.

Mitochondrial $1,25(OH)_2D_3$ production

Mitochondria were prepared from vitamin D deficient chick kidneys by standard differential centrifugation techniques. Kidneys were removed,rapidly minced and homogenised in 10 volumes of ice-cold buffer of 250 mM sucrose, 1 mM EGTA, 0.1% defatted BSA, 10 mM Hepes/KOH pH 7.4, in a Potter-Elvehjem homogenizer. The supernatant from a 400 G x 10 min,4°C centrifugation was pooled with a second supernatant prepared from a further homogenization of the pellet. The combined supernatants were spun at 8,000 G x 5 min, 4°C and the resulting pellet was gently resuspended and washed twice in ice-cold buffer of 120 mM KCl, 5 mM Na succinate, 2 mM K_2HPO_4, 1 mM $MgCl_2$ 1 µM rotenone, 10 mM Hepes/KOH pH 7.2. These mitochondria showed satisfactory coupling of oxidative phosphorylation (with respiratory control ratios of 5, and P/O ratios of close to 2) and energy-dependent calcium uptake.

Conversion of tritiated $25(OH)D_3$ to $1,25(OH)_2D_3$ was measured in identical 1.5 ml aliquots of mitochondrial suspension at 37°C over 4 minutes, using the extraction and separation procedures described for tubules.

Vitamin D Transport

Vitamin D transport studies were performed in isolated vitamin D-deficient chick tubules prepared as before, using a modification of a rapid cell fractionation technique described by Reinhart et. al. (17). Aliquots of isolated tubules were preincubated with the agent under study (or vehicle only) for 20 min at 37°C,exposed to tritiated $25(OH)D_3$ for times indicated, then rapidly homogenised in 10 volumes of an ice-cold buffer of 250 mM sucrose, 1 mM EGTA, 1% chick serum, 10 mM Hepes/KOH, pH 7.4. Chick serum was found to be effective in preventing post-homogenisation uptake of free $25(OH)D_3$ by mitochondria, presumably because of the presence of serum vitamin D-binding protein.

Aliquots of 1ml of homogenate were layered onto the surface of 40% Percoll (density 1.080gm/ml) preparations, isosmotic with homogenisation buffer, in Corex tubes. Tubes were then centrifuged at 30,000g x 30 secs. (excluding braking time), resulting in a cytosol-medium layer, including membranes and lighter organelles on the surface, and a mitochondrial enriched pellet. Mitochondrial and cytosol-medium fractions, and cell homogenates were extracted and analysed for vitamin D metabolites as above.

Marker enzymes

Succinate-cytochrome C reductase was performed by the method of King, (18) glucose-6-phosphatase according to Baginski (19), and 5'-nucleotidase was assayed by a kit purchased from Sigma Chemical Co.

Materials

Vitamin D metabolite standards were a kind gift of Dr. M. Uskokovic, Hoffman-La Roche, Nutley, N.J. U.S.A. Tritiated $25(OH)D_3$ was obtained from the Radiochemical Centre, Amersham U.K. and made up in ethanol. Collagenase 150 U/mg was from Worthington Freehold, N.J. U.S.A. Vinblastine sulphate was from John Bull Laboratories, Melbourne, Australia and 10 mM stock was made up in water. Cytochalasin was obtained from Sigma Chemical Co. St. Louis, Mo. USA, and a 10 mM stock was made up in DMSO:Ethanol 1:10(v/v). Percoll was obtained from Pharmacia Fine Chemicals Uppsala, Sweden, and protein estimations were performed using the Bradford reagent from Biorad Lab. CT. U.S.A. Statistical comparisons were made within experiments by the Students unpaired t test.

RESULTS

The microfilament antagonist, cytochalasin B, and the microtubule antagonist, vinblastine, inhibited the production of $1,25(OH)_2D_3$ from $25(OH)D_3$ by isolated vitamin D-deficient chick renal tubules in a dose dependent manner (Fig. 1). Cytochalasin D also inhibited the production of $1,25(OH)_2D_3$ production in a

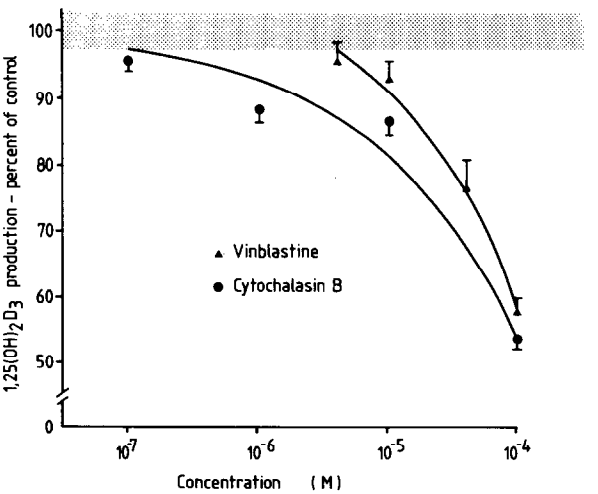

Figure 1. Cytoskeletal antagonists and $1,25(OH)_2D_3$ production by isolated tubules. Isolated vitamin D-deficient chick renal tubules were preincubated with the agents indicatd at 37°C for 20 minutes, then exposed to 50 nM of tritiated $25(OH)D_3$ for a further 20 minutes before extraction and separation of the vitamin D metabolites. Control $1,25(OH)_2D_3$ production was 10 pmol/mg tubule protein. 20 min. Points represent the mean percent of control $1,25(OH)_2D_3$ production, with standard errors indicated, for 4 observations.

similar fashion, and other microtubule antagonists, colchicine and deuterium oxide were also potent inhibitors (results not shown).

Production of the alternative metabolite, $24,25(OH)_2D_3$, by vitamin D-replete chick tubules was measured to assess the specificity of the action of cytoskeletal antagonists on vitamin D metabolism. Cytochalasin B at 100 μM and vinblastine at 50 μM did not inhibit production of this metabolite (data not shown).

A direct effect of cytoskeletal antagonists on the mitochondrial 1-α-hydroxylase enzyme was examined in isolated renal mitochondria. Mitochondrial production of $1,25(OH)_2D_3$ was not inhibited significantly by cytochalasin B 100 μM or vinblastine 50 μM; concentrations at which significant inhibition was observed in intact cells (data not shown).

Further investigation of vitamin D metabolite transport and the effects of cytoskeletal antagonists were performed by the rapid separation of tubule cell homogenates into "mitochondrial-rich" and "cytosol-medium" fractions on a Percoll gradient. Distribution of marker enzyme activities; succinate-cytochome C reductase, glucose-6-phosphatase and 5'-nucleotidase, for mitochondria endoplasmic reticulum, and plasma membranes respectively are shown (Table 1).

A mitochondrial fraction prepared from tubules preincubated with cytochalasin B 100 μM, and exposed to tritiated $25(OH)D_3$ 50nM for various times before homogenization, contained more tracer than control mitochondria at all time points (Fig.2). While net tracer activity of control mitochondria had

Table 1
Marker enzyme activities in Percoll Fractitionated Chick Tubule Homogenates

Enzyme	Fraction		
	Cell homogenate	Medium-cytosol	Mitochondria
succinate -cytochrome C reductase ($\frac{\mu mols\ succinate\ oxidised}{min.\ mg\ protein}$)	12.4	7.3	23.0
glucose-6-phosphatase ($\frac{\mu mols\ Pi\ liberated}{min.\ mg\ protein}$)	0.45	0.30	0.59
5'-nucleotidase ($\frac{\mu gm\ Pi\ liberated}{hour.\ mg\ protein}$)	29.3	32.7	4.7

An aliquot of isolated tubule homogenate was centrifuged at 30,000 xg for 30 seconds on Percoll, density 1.080 gms/l, to produce a "medium-cytosol" supernatant and a "mitochondrial" pellet. Fractions were separated and stored at -20°C until dilution and assay.

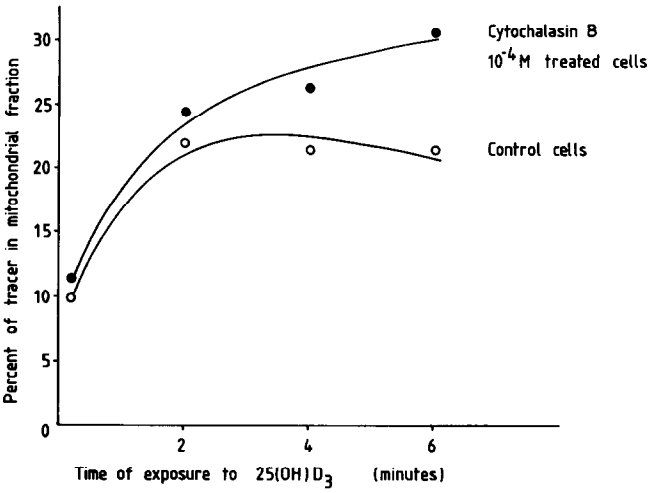

Figure 2. Uptake of [^{3}H]25(OH)D_3 by mitochondria in isolated chick renal tubules. Isolated tubules, preincubated with cytochalasin B 100 μM or vehicle, were exposed to labelled 25(OH)D_3 50 nM for the times indicated, then homogenized and a mitochondrial fraction was rapidly prepared as described in Methods. The total isotope activity of this fraction, expressed as a percentage of recovered isotope was plotted against exposure time. Points represent determinations from separate incubations randomized in time.

reached a plateau after several minutes, mitochondria from cytochalasin B treated tubules appeared to be further accumulating tracer.

Extraction and separation of metabolites from homogenates and cell fractions prepared from vitamin D-deficient tubules exposed to labelled 25(OH)D_3 50nM for 3 min, was performed. Cells preincubated with cytochalasin B 100 μM or vinblastine 50 μM were found to have increased 25(OH)D_3 content in cytosol-medium and mitochondrial fractions compared to controls. (Fig. 3). Both cytoskeletal antagonists caused a concomitant suppression of 1,25$(OH)_2D_3$ appearance in homogenates and cytosol-medium cell fractions. However the 1,25$(OH)_2D_3$ content of the mitochondrial fraction was found to be increased by pre-exposure of cells to cytochalasin B or vinblastine (Fig. 3).

DISCUSSION

The alkaloid fungal metabolites, the cytochalasins, are widely used as antagonists of microfilament mediated processes, including emiocytosis of secretory granules, endocytosis, cytokinesis and cell contraction and movement (13,14). Cytochalasins exhibit high affinity binding to cell actin and reversibly prevent polymerization of actin monomers (13,20). Vinblastine, a vinca alkaloid microtubule disrupter, binds to tubulin subunits and is used to antagonize microtubule-mediated functions, including hormone secretion, and nuclear division (21).

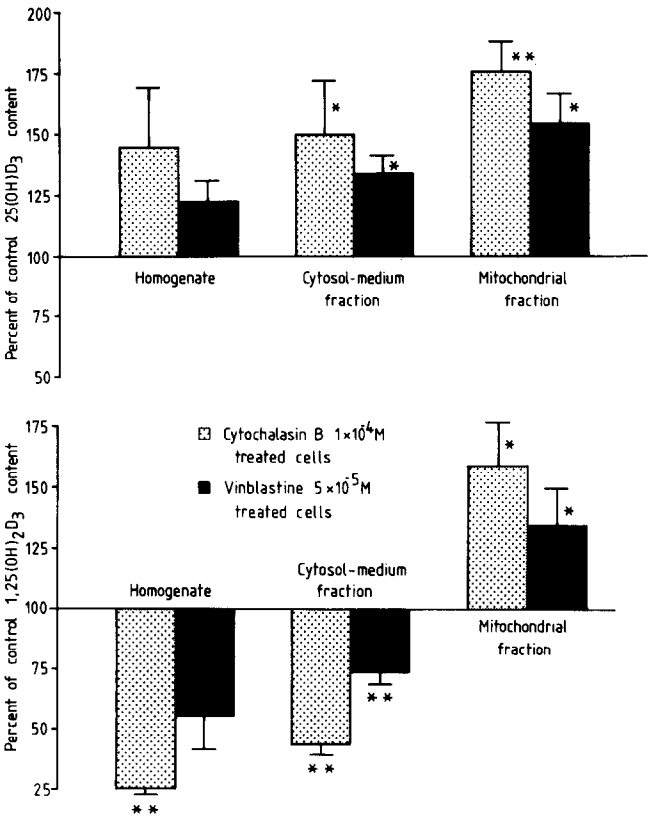

Figure 3. Content of $25(OH)D_3$ and $1,25(OH)_2D_3$ in fractionated renal tubules. Isolated tubules, preincubated with cytochalasin B 100 μM or vinblastine 50 μM for 20 minutes were exposed to $[^3H]25(OH)D_3$ for 3 minutes, then rapidly homogenised and fractionated as described in Methods. The fractions were extracted, vitamin D metabolites separated, and metabolite content calculated, using the known specific activity of the labelled $25(OH)D_3$. Columns represent the mean percent of control vitamin D metabolite content, with standard errors indicated, for four observations. *P<0.05 compared to control. **P<0.01 compared to control.

The cytoskeletal antagonists, cytochalasin B and vinblastine, inhibited the conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by isolated vitamin D-deficient chick renal tubules in dose-dependent fashion. These agents failed to affect production of $1,25(OH)_2D_3$ by isolated renal mitochondria at concentrations markedly inhibitory in tubule cells. This observation excludes a significant toxic effect on the 1α-hydroxylase enzyme itself, and is consistent with an action of these agents on vitamin D transport in the intact cell. The lack of effect of cytoskeletal antagonists on the production of $24,25(OH)_2D_3$ from vitamin D-replete tubules, while further supporting the specificity of their actions on $1,25(OH)_2D_3$ production, does not preclude an action on an important transport step. Vitamin D metabolite transport may only be rate-limiting in vitamin D deficient tubules, or for the 1α-hydroxylase mediated conversion.

A rapid isolation procedure for preparation of a mitochondrial fraction from homogenates of tubule cells was developed by a modification of Reinhart et. al. (17). A single Percoll concentration of 40%, density 1.080gm/l, isosmotic with homogenisation buffer, was found to best balance the conflicting demands of purity and recovery. This method failed to separate microsomes from mitochondria. However, in a single, rapid centrifugation step it produced a reasonably enriched mitochondrial fraction, comparable to differential centrifugation techniques, and substantially free of plasma membranes and medium. Time course studies using labelled $25(OH)D_3$ confimed a progressive uptake of tracer, reaching a plateau of net mitochondrial tracer activity presumably reflecting a steady state turnover of vitamin D metabolite.

Surprisingly, net tracer content of a mitochondrial fraction of cells exposed to labelled $25(OH)D_3$ was increased by pre-exposure of the cells to cytochalasin B, and appeared to continue to diverge from the net control tracer activity when that had reached steady-state.

Analysis of the metabolite content revealed that the cell homogenates and cytosol-medium fractions from cytoskeletal antagonist-treated cells contained less $1,25(OH)_2D_3$ and concomitantly raised $25(OH)D_3$. This might be expected from agents that inhibit conversion by any mechanism. However, both $25(OH)D_3$ and $1,25(OH)_2D_3$ content was increased in the mitochondrial fraction of tubules preincubated with cytochalasin B or vinblastine, and exposed to labelled $25(OH)D_3$ for 3 minutes. These observed effects of cytoskeletal antagonists are strongly suggestive of a role for the cytoskeleton in the transport of newly synthesized $1,25(OH)_2D_3$ out of the mitochondrion and through the cell.

Production of $1,25(OH)_2D_3$ is known to be inhibited by physiological concentrations of $1,25(OH)_2D_3$ itself, in tubules by a process requiring DNA transcription (22) and obviously not applicable over this time course. An acute inhibitory effect of exogenous $1,25(OH)_2D_3$ on its production by kidney homogenates, has been demonstrated, albeit at high concentrations (23). Nevertheless, an action of cytoskeletal antagonists to cause an acute accumulation of newly synthesized $1,25(OH)_2D_3$ in an important mitochondrial compartment may explain the inhibitory effect of antagonists on net tubule $1,25(OH)_2D_3$ production. Steroid product-inhibition of hydroxylase activity is well recognized in certain steroid producing cells (24,25), and several examples of rapid nucleus-independent effects of steroid hormones have been characterized (26).

While cytochalasin B has well documented inhibitory effects on hexose transport, (27) we obtained similar results using cytochalasin D, which does not affect glucose uptake. Cytochalasins do not inhibit cell oxygen consumption (28) or amino acid uptake, protein, DNA or ATP synthesis (4,29). Vinblastine has been reported to have microtubule-independent effects of questionable significance,

namely precipitation of actin at very high concentrations (30) and inhibition of calmodulin dependent phosphodiesterase (31). The former action may cause actions of vinblastine to "cross-react" with those of cytochalasin, but as microtubule disassembly is calmodulin dependent (32), vinblastine actions on microtubules cannot be mediated through an inhibitory effect on cell calmodulin.

The conversion of cholesterol to pregnenolone in the mitochondria of certain steroid secreting cells is the rate-limiting step, sensitive to trophic peptide hormones and cyclic AMP, and catalysed by a side-chain-cleavage steroid hydroxylase. This enzyme is a cytochrome P_{450} mixed function oxidase similar to the renal 1α-hydroxylase (1,2). Evidence has been provided that acute ACTH and LH stimulated steroidogenesis in adrenal, and Leydig or granulosa cells respectively is caused by increasing transport of cholesterol to the mitochondrion (6,8,10,11). Furthermore studies using cytochalasin B (3,6,7,9,10,12) and anti actin antibody (4,5) have demonstrated that microfilament-mediated transport of cholesterol is a rate-limiting step for this steroidogenic response. In adrenal cells and using similar concentrations of vinblastine, a role for the microtubule system also in ACTH and cyclic AMP stimulated cholesterol transport was postulated (7). It seems likely that in transport, as in other functions, the elements of the cytoskeleton act as an integrated unit (14).

Of particular relevance to this study is the observation that in the presence of the trophic hormone ACTH, accumulated cholesterol is transported away from the adrenal cell mitochondrion (11), and that this transport is also inhibited by cytochalasin B and vinblastine (7).

Observed effects on vitamin D metabolism by cytoskeletal disrupters in kidney cells in-vitro do not prove a role for the cytoskeleton in the control of renal vitamin D metabolism in-vivo. However the data presented, together with the analogy of the cytoskeletally mediated steroidogenic responses discussed, suggest a role for the cytoskeleton in the transport of vitamin D metabolites within the renal tubule cell. The nature of this transport, and its function in regulation of $1,25(OH)_2D_3$ production must be the subject of further study.

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