Microtubules Mediate Cellular 25-Hydroxyvitamin D₃ Trafficking and the Genomic Response to 1,25-Dihydroxyvitamin D₃ in Normal Human Monocytes

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The most active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), is a potent steroid hormone. Similar to other steroids, its genomic actions require binding to its intracellular receptor and interaction of the 1,25(OH)₂D₃-vitamin D receptor (VDR) complex with specific vitamin D responsive regions in the genome (1, 2).

It has been suggested recently that hormone-free glucocorticoid receptors are located predominantly in the cytoplasm, and, after the addition of steroid, they are rapidly translocated to the nucleus (3–6). The transfer of glucocorticoid receptor into the nucleus involves translocation along microtubules as revealed by immunofluorescent studies (7) in a process that is driven by tubulin-associated dynein motors (8).

In the case of vitamin D, there is some controversy as to whether apoVDRs reside only in the nucleus like the thyroid hormone receptor (9) or whether they can undergo ligand-dependent translocation like the glucocorticoid receptor (10, 11). Using a recently developed fluorescent ligand, Barsony et al. (12) were able to demonstrate the cytoplasmic localization of the VDR in viable human skin fibroblasts, porcine kidney epithelial cells, human breast cancer cells, and rat osteosarcoma cells, supporting previous immunocytochemical findings in fixed human fibroblasts (13) and osteoblasts (14). Although immunocytochemistry has shown that cytoplasmic VDR colocalizes with tubulin and that disruption of microtubular assembly blocks the translocation of the 1,25(OH)₂D₃-VDR complex into the nucleus (15) in fixed fibroblasts, the role of microtubules on VDR transport in viable cells has never been evaluated. We hypothesized that if this intracellular transport system is of physiological relevance, the genomic response to 1,25(OH)₂D₃ should be impaired with alterations in the structure or function of the microtubule network. We tested this hypothesis in normal human monocytes.

Human monocytes express receptors for 1,25(OH)₂D₃ that are indistinguishable from those described in classical 1,25(OH)₂D₃ target tissues (16), and the interactions of 1,25(OH)₂D₃ with monocytes-macrophages have critical implications for the regulation of immune responses (17–20). Our laboratory has demonstrated that peripheral blood monocytes from normal individuals constitutively express 1α-hydroxylase, the enzyme responsible for the conversion of 25-hydroxyvitamin D₃ (25(OH)D₃) to 1,25(OH)₂D₃ (21). We have also shown that when peripheral blood monocytes were exposed to physiological concentrations of 1,25(OH)₂D₃, 1α-hydroxylase activity is markedly suppressed. In addition, exogenous 1,25(OH)₂D₃ promotes an induction of vitamin D catabolism by increasing 24-hydroxylase mRNA² and activity (22). Because both effects of the steroid require at least 2 h of exposure to 1,25(OH)₂D₃ (22), it is likely that the inhibition of 1,25(OH)₂D₃ production by 1,25(OH)₂D₃ also involves a genomic mechanism. In the present studies, we used this human monocyte model to assess the physiological relevance of microtubule integrity in the response to 1,25(OH)₂D₃. This report demonstrates for the first time that integrity of the microtubule network is critical for a normal genomic response to 1,25(OH)₂D₃ and that an intracel-

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lular tubulin-25(OH)D₃ transport system mediates the delivery of 25(OH)D₃ to mitochondria, thus modulating the rate of 1,25(OH)₂D₃ synthesis by monocytes.

**EXPERIMENTAL PROCEDURES**

Materials—1,25(OH)₂D₃ was kindly provided by Dr. Milan Uskokovic (Hoffman-La Roche). 1,25-Dihydroxy[26,27-3H]cholecalciferol (specific activity, 120-174 Ci/mmol) and 25-hydroxy[26(27)-3H]cholecalciferol (specific activity, 28 Ci/mmol) were obtained from Amersham. Colchicine, vinblastine, isocitrate, and NADPH were obtained from Sigma.

Culture of Human Monocytes—Peripheral blood was obtained from normal volunteers by venipuncture. Mononuclear leukocytes were isolated using a Ficoll-Hypaque gradient (Pharmacia Biotech Inc.). Cells were plated in six-well plates at a concentration of 7 × 10⁵ cells per well in 1 ml of RPMI 1640 containing 1% fatty-acid-free albumin, 50,000 units/filser penicillin G sodium, 50,000 μg/ml streptomycin sulfate, 10 mM HEPES, and 0.8 mM NaHCO₃. After an incubation period of 18 h at 37 °C, media and nonadherent cells were removed, and adherent cells were washed (once with 2 ml of phosphate-buffered saline (PBS) and twice with 1 ml of RPMI 1640 containing 0.1% fatty-acid-free albumin). More than 95% of the adherent cells stained positively for macrophage-specific α-naphthyl acetate esterase activity (23). This adherent cell population was used in all studies.

This adherent cell population was used in all studies. The effect of the reversible microtubule-disrupting agents nocodazole and vinblastine on 1,25(OH)₂D₃ synthesis and the stimulation of vitamin D catabolism by monocytes was measured. The vitamin D metabolite fraction eluting with the methanol strip from wells incubated with 25(OH)D₃ was quantified using a ribonuclease protection assay (24) and was 32P-labeled with [32P]CTP (Amersham). The size of the 32P-labeled transcript was 150-nt homology with human glyceraldehyde-3-phosphate dehydrogenase (25) and the antisense control template for human glyceraldehyde-3-phosphate dehydrogenase (26). The vitamin D metabolite fraction eluting with the methanol strip from wells incubated with 1,25(OH)₂D₃ was stripped with 4 ml of toluene:isooctane (1:1) as the solvent.

**Total Cellular Uptake of 1,25(OH)₂D₃ or 25(OH)D₃ by Human Peripheral Monocytes**—A modification of the procedure reported by Keenan and Holmes (26) was used. One milliliter of fresh incubation media containing tritiated vitamin D metabolites was added to adherent cells. To prepare this media, an ethanolic solution of [3H]1,25(OH)₂D₃ or [3H]25(OH)D₃ was added to rat liver microsomal fractions in ethanol:water (1:1), and the remaining solution was added to the incubation media to minimize the loss of vitamin D metabolites to glassware and culture dishes. Total concentration of ethanol was less than 0.1%, and final concentration of ethanol was 0.78%. The radioactivity in the incubation media was quantified by liquid scintillation counting. Wells were washed once with 2 ml of acetonitrile:water (1:1), and radioactivity eluting from the supernatants was quantified using a scintillation counter (ICN Micromedic System Inc., Huntsville, AL).

**Role of Microtubules in Vitamin D Transport and Action**—Adherent cells were exposed to 0 or 10 nM 1,25(OH)₂D₃ for 4 h. Nuclear and cytoplasmic preparations were collected. Nuclei were extracted with 0.78 nM, respectively. Preliminary experiments demonstrated that total cellular uptake of 1,25(OH)₂D₃ or 25(OH)D₃ reached a plateau after a 30-min incubation. Total cellular uptake of both sterols was measured in untreated and colchicine-treated (750 μM for 30 min) monocytes. After a 1-h incubation of monocytes at 4 or 37 °C with tritiated 1,25(OH)₂D₃ or 25(OH)D₃ medium was removed and cells were washed twice with ice-cold PBS and lysed with 0.1 M NaOH containing 1% fatty-acid-free albumin until fixation. For nocodazole treatment, monocytes were exposed to 0 or 10 μM nocodazole for 30 min at 37 °C. Cells were washed twice with PBS and incubated in fresh RPMI 1640 containing 1% fatty-acid-free albumin until fixation. For nocodazole treatment, monocytes were exposed to 0 or 10 μM nocodazole for 30 min at 37 °C and then fixed in paraformaldehyde (4%) for 20 min at room temperature. Immunofluorescence Staining for α-Tubulin—Peripheral blood mononuclear leukocytes were plated on coverslips in six-well plates following the protocols described for monocyte isolation. Adherent cells were exposed to 0, 10, 25, or 750 μM colchicine for 30 min at 37 °C. Cells were washed twice with PBS and incubated in fresh RPMI 1640 containing 1% fatty-acid-free albumin until fixation. For nocodazole treatment, monocytes were exposed to 0 or 10 μM nocodazole for 30 min at 37 °C and then fixed in paraformaldehyde (4%) for 20 min at room temperature. Immunofluorescence Staining for α-Tubulin—Peripheral blood mononuclear leukocytes were plated on coverslips in six-well plates following the protocols described for monocyte isolation. Adherent cells were exposed to 0, 10, 25, or 750 μM colchicine for 30 min at 37 °C. 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free albumin, then twice with PBS alone and placed on ice. After the addition of 2 ml of TEDK (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 400 mM KCl, and 5 mM dithiothreitol), the contents of each well were sonicated for 30 s. Samples were transferred to small tubes, and 500 μl of charcoal was added. Tubes were kept on ice for 15 min and centrifuged at 2500 rpm for 15 min. Aliquots (0.5 ml) of the supernatants were mixed with 5.0 nM [3H]25(OH)D3 dissolved in 200 μl of ethanol fraction. Organic solvents were air-dried at room temperature.

Subcellular 25(OH)D3 Uptake—Subcellular uptake of 25(OH)D3 was measured using a modification of the procedure described by Shany et al. (27). Adherent cells were incubated with or without colchicine (10 and 750 μM). After a 30-min incubation at 37°C, cells were washed and incubated at 37°C for 1 h in 1 ml of RPMI 1640 containing 0.1% fatty-acid-free albumin. 0.1 μlCi of [3H]25(OH)D3 and 10 μM ketoconazole, a cytochrome P-450 inhibitor, to block 25(OH)D3 metabolism. Cells were rinsed once with phosphate-buffered saline and twice with RPMI 1640 containing 0.1% fatty-acid-free albumin. Monocytes were disrupted by sonication in 1 ml of TMSS buffer, pH 7.4 (15 mM Tris acetate, 2 mM MgCl2, 2.5 mM succinate, and 250 mM sucrose) containing phenylmethylsulfonyl fluoride (10 μM). Fifteen wells of untreated and colchicine-treated sonicated monocytes were pooled in 50-ml culture tubes, and final volumes were measured. 0.5 ml of these samples were counted for tritium, and total tritium in whole cell homogenates was calculated. For subcellular fractionation, homogenates were divided in 4 aliquots of equal volume. Aliquots were centrifuged at 700 × g for 10 min at 4°C. The supernatants were centrifuged at 10,000 × g for 10 min at 4°C. Final supernatants were centrifuged at 100,000 × g for 1 h at 4°C. The resultant pellets (700 × g, containing mainly nuclei and cellular membranes; 8,500 × g, mainly mitochondrial fraction; 10,000 × g, residual mitochondrial fraction; 100,000 × g, mainly microsomal fraction) were resuspended in 0.5 ml of ethanol, vortexed, and transferred to miniscintillation vials. Potential residual pellets were washed three times in 1 ml of hexaneisopropanol alcohol (95:5) and pooled with the ethanol fraction. Organic solvents were air-dried at room temperature, and the amount of tritium associated to each subcellular fraction was counted.

To assess the purity of the subcellular fractions, we assayed succinic dehydrogenase (28) and NADPH-cytochrome c reductase (29) activities as mitochondrial and microsomal specific markers, respectively. Results were expressed as percent of total enzymatic activity in each subcellular fraction.

**RESULTS**

Effect of Microtubule Disruption on the Response of Normal Human Monocytes to 1,25(OH)2D3—We examined the effect of disruption of microtubular assembly on the ability of 1,25(OH)2D3 to suppress its own production and to induce vitamin D catabolism. Fig. 1 shows that, in normal human monocytes, the ability of 1,25(OH)2D3 to inhibit its own synthesis was impaired by colchicine in a dose-dependent manner. At a concentration of colchicine of 25 μM, 1,25(OH)2D3 was no longer able to suppress monocyte 1,25(OH)2D3 production. The number of monocytes that stained positive for polymerized tubulin decreased from 91% in control preparations to 62, 22, and 9% after a 30-min exposure to concentrations of colchicine of 10, 25, and 750 μM, respectively.

Because colchicine alone caused a dose-dependent reduction of 1,25(OH)2D3 production by monocytes, we performed the experiments summarized in Fig. 2 to define the effects of microtubule disruption in the response of monocytes to 1,25(OH)2D3. The lowest (25 μM) and highest (750 μM) doses of colchicine, effective in blocking the ability of 1,25(OH)2D3 to suppress its own production, were used to examine the effect of microtubule disruption on the ability of 1,25(OH)2D3 to suppress its own synthesis (upper panel) and to induce vitamin D catabolism (generation of 24-hydroxylated metabolites) more polar than 1,25(OH)2D3 (polar metabolites; lower panel). In monocytes with intact microtubules (Controls), exposure to 0.24 nm 1,25(OH)2D3 for 4 h reduced 1,25(OH)2D3 synthesis to 55.2 ± 1.3% and increased the generation of polar metabolites 7-fold above vehicle controls, respectively. However, when monocytes were treated with 25 or 750 μM colchicine for 30 min before exposure to 0 (vehicle) or 0.24 nm 1,25(OH)2D3 for 4 h (Fig. 2, Pre) exogenous 1,25(OH)2D3 could no longer reduce 1,25(OH)2D3 synthesis nor enhance the production of polar metabolites. As mentioned for the dose response to colchicine, in monocytes exposed to colchicine alone (vehicle), there was a marked reduction of 1,25(OH)2D3 synthesis.

To assess whether the lack of response to 1,25(OH)2D3 with colchicine treatment was the result of a direct effect of colchicine on monocyte hydroxylases rather than the consequence of a defective access of the 1,25(OH)2D3-VDR complex to the nucleus, monocytes were first incubated with 0 (vehicle) or 0.24 nm 1,25(OH)2D3 for 4 h and then exposed to colchicine for 30 min. Measurements of synthesis of 1,25(OH)2D3 and polar metabolites (Fig. 2, Post) show, in vehicle controls, a similar decrease in 1α-hydroxylase as described in Pre experiments with no significant changes in the apparent activity of the hydroxylases involved in vitamin D catabolism. However, in these protocols, microtubule disruption occurred after the 1,25(OH)2D3-VDR complex had interacted with the genome, and, despite the reduction in 1α-hydroxylase activity in vehicle control monocytes, exogenous 1,25(OH)2D3 could reduce its own synthesis and increase vitamin D catabolism with a potency similar to that observed in monocytes with intact microtubules. Specifically, a 54.2 ± 3.1% reduction of 1,25(OH)2D3 synthesis and 743.2 ± 24.1% induction of vitamin D catabolism by 0.24 nm 1,25(OH)2D3 was observed in the presence of 25 μM colchicine on monocyte hydroxylases rather than the consequence of a defective access of the 1,25(OH)2D3-VDR complex to the nucleus, monocytes were first incubated with 0 (vehicle) or 0.24 nm 1,25(OH)2D3 for 4 h and then exposed to colchicine for 30 min. Measurements of synthesis of 1,25(OH)2D3 and polar metabolites (Fig. 2, Post) show, in vehicle controls, a similar decrease in 1α-hydroxylase as described in Pre experiments with no significant changes in the apparent activity of the hydroxylases involved in vitamin D catabolism. However, in these protocols, microtubule disruption occurred after the 1,25(OH)2D3-VDR complex had interacted with the genome, and, despite the reduction in 1α-hydroxylase activity in vehicle control monocytes, exogenous 1,25(OH)2D3 could reduce its own synthesis and increase vitamin D catabolism with a potency similar to that observed in monocytes with intact microtubules. Specifically, a 54.2 ± 3.1% reduction of 1,25(OH)2D3 synthesis and 743.2 ± 24.1% induction of vitamin D catabolism by 0.24 nm 1,25(OH)2D3 was observed in the presence of 25 μM colchicine on monocyte hydroxylases rather than the consequence of a defective access of the 1,25(OH)2D3-VDR complex to the nucleus, monocytes were first incubated with 0 (vehicle) or 0.24 nm 1,25(OH)2D3 for 4 h and then exposed to colchicine for 30 min. Measurements of synthesis of 1,25(OH)2D3 and polar metabolites (Fig. 2, Post) show, in vehicle controls, a similar decrease in 1α-hydroxylase as described in Pre experiments with no significant changes in the apparent activity of the hydroxylases involved in vitamin D catabolism. However, in these protocols, microtubule disruption occurred after the 1,25(OH)2D3-VDR complex had interacted with the genome, and, despite the reduction in 1α-hydroxylase activity in vehicle control monocytes, exogenous 1,25(OH)2D3 could reduce its own synthesis and increase vitamin D catabolism with a potency similar to that observed in monocytes with intact microtubules. Specifically, a 54.2 ± 3.1% reduction of 1,25(OH)2D3 synthesis and 743.2 ± 24.1% induction of vitamin D catabolism by 0.24 nm 1,25(OH)2D3 was observed in the presence of 25 μM colchicine.
with 750 μM colchicine, an reversible microtubule disrupting agent. Fig. 4 shows that inability of 1,25(OH)2D3 to induce 24-hydroxylase mRNA expression in monocytes was observed with another microtubule disrupting agent, vinblastine, at 50 and 100 μM colchicine and, similar to the highest dose of colchicine (750 μM) followed the 4-h exposure to 0.24 nM 1,25(OH)2D3 for 4 h. Total RNA from monocytes was assayed for mRNA levels of 24-hydroxylase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using ribonuclease protection assay. A shows two representative gels, in monocytes from the same individual. B depicts the densitometric analysis of the 24-hydroxylase/GAPDH mRNA ratios. Values represent mean ± S.E. from 4 independent experiments performed in duplicate.

Similar blockage of the ability of exogenous 1,25(OH)2D3 to control 1,25(OH)2D3 production and vitamin D catabolism by monocytes was observed with another microtubule disrupting agent, vinblastine, at 50 and 100 μM concentrations (data not shown).

These results demonstrate that microtubular integrity is required for monocytes to respond to 1,25(OH)2D3. Further support for these findings came from measurements of steady state levels of 24-hydroxylase mRNA using a ribonuclease protection assay. Fig. 3 shows that intact monocytes responded to physiological concentrations of 1,25(OH)2D3 with a marked induction of 24-hydroxylase mRNA. However, pretreatment of monocytes with colchicine impaired the ability of 1,25(OH)2D3 to induce 24-hydroxylase gene transcription in a dose-dependent manner. A partial reduction of 24-hydroxylase mRNA levels was achieved with 10 μM colchicine and, similar to the increase in the synthesis of polar metabolites, 25 μM colchicine caused total inhibition of the 1,25(OH)2D3-mediated increase in 24-hydroxylase mRNA. When the 30-min treatment with colchicine (10, 25, and 750 μM) followed the 4-h exposure to 0.24 nM 1,25(OH)2D3, the increase in 24-hydroxylase mRNA levels was identical with that observed in monocytes exposed to 1,25(OH)2D3 alone. This suggests that a 30-min exposure to colchicine has no significant direct effect either in mRNA stability or in 1,25(OH)2D3-mediated transcription. To confirm that the effects of colchicine on the genomic action of 1,25(OH)2D3 in monocytes were mediated by microtubule disruption only, we performed similar experiments with nocodazole, a reversible microtubule disrupting agent. Fig. 4 shows that the inability of 1,25(OH)2D3 to induce 24-hydroxylase mRNA levels when microtubules are disrupted with 10 μM nocodazole (74% of nocodazole-treated monocytes stained negatively for polymerized tubulin) can be totally reversed if monocytes are allowed to recover microtubular integrity after removal of nocodazole from the incubation media.

Effect of Colchicine on 1,25(OH)2D3 Uptake and Formation of the 1,25(OH)2D3-VDR Complex—To characterize potential mechanisms involved in the lack of response to 1,25(OH)2D3 with microtubule disruption, we measured the effect of the highest dose of colchicine (750 μM) on 1,25(OH)2D3 uptake by monocytes. Colchicine did not affect total cellular uptake of 1,25(OH)2D3 after a 1-h incubation either at 4 °C (C: 1.5 ± 0.2; T: 1.6 ± 0.2 fmol/μg of DNA/h; n = 3) or at 37 °C (3.0 ± 0.3 versus 3.5 ± 0.2; n = 4).

We next examined whether colchicine affected the formation of the 1,25(OH)2D3-VDR complex. In three independent experiments performed in triplicate, there was no significant difference in maximal specific binding of 1,25(OH)2D3 to the VDR between untreated and colchicine-treated (750 μM) monocytes (C: 0.21 ± 0.02; T: 0.19 ± 0.01 fmol/μg of DNA, n = 3).

Effect of Microtubule Disruption on Monocyte 1α-Hydroxylase Activity—In Fig. 1, we showed that colchicine alone markedly reduced monocyte 1,25(OH)2D3 synthesis, even at a 10 μM concentration, a dose only partially effective in blocking the response to 1,25(OH)2D3. To examine whether this reduction on 1,25(OH)2D3 synthesis by colchicine was the result of impaired protein synthesis, a known side effect of colchicine treatment (30) that could lead to a nonspecific reduction in the amount of 1α-hydroxylase in monocytes, we measured the ef-
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Microtubule disruption affected intracellular 25(OH)D3 trans- 
port to the enzyme. Therefore, we measured 1α-hydroxylase activity in mitochondrial and microsomal fractions. We found no changes in succinate dehydrogenase activity in the mitochondrial fraction of untreated and colchicine-treated monocytes. We observed a concomitant increase in the amount of 25(OH)D3 in the cytosol and with no changes in the 25(OH)D3 associated to microsomal or nuclear fractions.

To test whether this reduction in the mitochondrial uptake of 25(OH)D3 could be the result of a decrease in the number of mitochondria with microtubule disruption, we measured succinate dehydrogenase activity in the mitochondrial fraction of untreated and colchicine-treated monocytes. We found no changes in succinate dehydrogenase activity (C: 0.022 ± 0.002; T: 0.020 ± 0.002 pmol/min/μg of DNA, n = 2) with colchicine treatment.

Subcellular Localization of Monocyte 1α-Hydroxylase—The previous findings also suggested the mitochondrial localization of monocyte 1α-hydroxylase. Therefore, we measured 1α-hydroxylase activity in mitochondrial and microsomal fractions. In mitochondria, 1,25(OH)2D3 production was 0.68 ± 0.07 fmol/μg of DNA/h, whereas in microsomes, 1,25(OH)2D3 synthesis was undetectable (0.01 ± 0.01 fmol/μg of DNA/h).

![Image](91x446 to 251x742)

**FIG. 5.** Effect of nocodazole on monocyte 1α-hydroxylase activity. Monocytes were incubated with 0 or 10 μM nocodazole for 30 min, and 1α-hydroxylase activity was measured as outlined under “Experimental Procedures.” To examine the reversibility of nocodazole effects on 1,25(OH)2D3 production (reversed), after the 30-min exposure to 0 or 10 μM nocodazole, nocodazole was removed and monocytes were allowed to recover microtubular assembly for 4 h before assaying 1α-hydroxylase activity.

**TABLE I**

Kinetic parameters of the 1α-hydroxylase of human monocytes

|        | Km (nm) | Vmax (fmol/μg DNA·h) |
|--------|---------|---------------------|
| Control (n = 5) | 17.8 ± 2.2 | 32.1 ± 2.4 |
| Colchicine, 10 μM (n = 2) | 30.2 ± 3.1* | 34.5 ± 3.0 |
| Colchicine, 750 μM (n = 3) | 52.2 ± 1.7* | 32.1 ± 2.1 |

* Differ from control, p ≤ 0.01.

![Image](318x383 to 539x578)

![Image](251x742 to 501x983)
TABLE II
Enzymatic characterization of subcellular fractions

Succinate dehydrogenase and NADPH-cytochrome c reductase activities were measured as specific mitochondrial and microsomal markers, respectively. Results are expressed as percent of total enzymatic activity. Values are mean ± S.E. of three independent experiments.

| Subcellular Fraction     | Succinate dehydrogenase | NADPH-cytochrome c reductase |
|--------------------------|-------------------------|-----------------------------|
| Mitochondria             | 70.2 ± 2.5              | 9.3 ± 1.1                   |
| Microsomes               | 17.5 ± 1.0              | 74.8 ± 4.8                  |
| Nuclei + membranes       | 7.8 ± 1.2               | 12.0 ± 2.1                  |
| Cytosol                  | 5.8 ± 1.0               | 4.9 ± 1.1                   |

TABLE III
Effect of colchicine on subcellular 25(OH)D₃ uptake by human monocytes

Monocytes were exposed to 0, 10, or 750 μM colchicine for 30 min, washed, and incubated for 1 h with 0.1 μCi of [³H]25(OH)D₃. Cells were sonicated for 30 s, and we measured 25(OH)D₃ associated with each subcellular fraction obtained by differential centrifugation. Results were expressed as percent of total recovered tritium. Values are mean ± S.E. of three to five independent experiments performed in triplicate.

| Subcellular Fraction     | Control     | Colchicine (10 μM) | Colchicine (750 μM) |
|--------------------------|-------------|---------------------|---------------------|
| Nuclei + membranes       | 19.3 ± 2.2  | 20.2 ± 2.8          | 21.0 ± 1.8          |
| Mitochondria             | 43.7 ± 3.2  | 33.2 ± 2.2*         | 31.9 ± 2.6*         |
| Microsomes               | 10.1 ± 0.6  | 9.7 ± 0.8           | 9.2 ± 0.3           |
| Cytosol                  | 27.3 ± 2.8  | 36.4 ± 1.4*         | 38.2 ± 2.2*         |

* p ≤ 0.05 from controls.

Clearly, monocyte 1α-hydroxylase, like the renal enzyme, is located exclusively in mitochondria.

To determine whether the reduction of 1,25(OH)₂D₃ production by colchicine involved reduced 25(OH)D₃ transport to the mitochondria or directly impaired mitochondrial 25(OH)D₃ uptake, we measured 1α-hydroxylase activity in (a) mitochondrial fractions isolated from intact and colchicine-treated monocytes and (b) in isolated mitochondrial fractions from intact monocytes in the presence of 0 or 10 μM colchicine. In both protocols, we found no effect of colchicine on mitochondrial 1,25(OH)₂D₃ production (a control: 0.65 ± 0.12 fmol/μg of DNA/h; colchicine: 0.63 ± 0.09, n = 2; (b) control: 0.68 ± 0.07; colchicine: 0.63 ± 0.08 fmol of 1,25(OH)₂D₃/μg of DNA/h).

DISCUSSION

1,25(OH)₂D₃ is the most active form of vitamin D₃. Its actions extend beyond calcium homeostasis to impact a variety of nonclassical targets including the immune system. To elicit a biological response, 1,25(OH)₂D₃, the most active form of vitamin D, is a potent steroid hormone. Its actions extend beyond calcium homeostasis and to induce 24-hydroxylase mRNA and activity in normal human monocytes. The lack of response to exogenous 1,25(OH)₂D₃ with microtubular disruption could not be attributed to a reduced uptake of 1,25(OH)₂D₃ by monocytes or to a defective formation of the VDR-1,25(OH)₂D₃ complex. Clearly, microtubular integrity is required after the formation of the 1,25(OH)₂D₃-VDR complex for monocytes to elicit a normal response to 1,25(OH)₂D₃. In addition, our studies could not demonstrate any direct action of microtubule disrupting agents on both genomic effects of 1,25(OH)₂D₃. The ability of exogenous 1,25(OH)₂D₃ to suppress monocyte 1,25(OH)₂D₃ production and to induce 24-hydroxylase mRNA and activity was not affected when the microtubule disrupting agent was added after exposure to the steroid for 4 h. These results in viable human monocytes support previous reports in fixed cells (13, 14) of cytoplasmic VDR localization. In contrast to gene activation by the glucocorticoid receptor (31), microtubule integrity is mandatory for 1,25(OH)₂D₃-VDR-mediated modulation of the transcription of vitamin D responsive genes. In addition to our findings in vitro, the recent clinical demonstration of a phenotype of vitamin D-resistant rickets type 1, caused by a defective nuclear translocation of an otherwise normal VDR (32), emphasizes the critical role of the tubulin transport system mediating cytoplasmic to nuclear VDR-1,25(OH)₂D₃ translocation in 1,25(OH)₂D₃ action.

The present studies also demonstrated that disruption of microtubular integrity significantly decreased the ability of human monocytes to synthesize 1,25(OH)₂D₃. Since colchicine was reported to decrease protein synthesis (30), we examined whether this reduction in 1,25(OH)₂D₃ generation was mediated by a decreased expression of monocyte 1α-hydroxylase. Kinetic analysis demonstrated that disruption of microtubular assembly did not affect the Vₘₐₓ of the enzyme but markedly reduced its apparent affinity for its substrate, 25(OH)D₃. Further characterization of the mechanisms mediating this increase in the Kₘ for 25(OH)D₃ showed that microtubule disruption did not affect whole cell uptake of 25(OH)D₃ but markedly decreased its intracellular delivery to the mitochondria, suggesting a role for microtubules in intracellular 25(OH)D₃ transport. We have also demonstrated that the reduction in mitochondrial 25(OH)D₃ uptake could not be attributed to a reduction in the number of mitochondria since succinate dehydrogenase activity was not decreased in colchicine-treated monocytes. The observation that 1α-hydroxylase activity in isolated mitochondria was not affected by a dose of colchicine that effectively reduced 1,25(OH)₂D₃ production by intact monocytes indicates no direct effect of colchicine on mitochondrial 25(OH)D₃ uptake or enzymatic activity. Thus, contrary to the well accepted theory of simple diffusion of 25(OH)D₃ due to its lipophilic nature, microtubules participate in the intracellular transport of 25(OH)D₃ to the mitochondrial 1α-hydroxylase of human monocytes. Similarly, cytoskeletal components were shown to mediate the transport of adrenal steroid precursors to the mitochondria, thus limiting the rate of synthesis of adrenal steroids (33). A mitochondrial localization of monocyte 1α-hydroxylase was also demonstrated in the chick myelomonocytic cell line HD11 (27), and the kinetics of the avian enzyme has marked similarities with the 1α-hydroxylase expressed in human pulmonary alveolar macrophages in sarcoidosis (34, 35).

Similar to our finding of impaired 1,25(OH)₂D₃ synthesis by monocytes with microtubular disruption, reduction in 1,25(OH)₂D₃ production by vinblastine was demonstrated in renal tubules from vitamin D-deficient chicks (36). However, for the avian renal enzyme, there was no actual reduction in total 1,25(OH)₂D₃ synthesized but an impaired exit of the steroid from the mitochondria and out of the renal epithelial cells. On the contrary, in our human monocyte model, we measured the total 1,25(OH)₂D₃ generated regardless of its subcellular or extracellular location, and we found a marked decrease in 1,25(OH)₂D₃ levels with microtubule disruption with no alteration in the Vₘₐₓ of the enzyme. Therefore, despite the similarities in subcellular localization and regulation of the 1α-
hydroxylase of human monocytes, it is dear that the effects of microtubular disruption on the intracellular transport of vitamin D metabolites vary with cell type, species, and vitamin D status.

If the cytoskeletal abnormalities reported for platelets in chronic uremia (37) are present in other cell types such as renal epithelia or peripheral monocytes, it is likely that a defective intracellular transport may partially explain the pathophysiological relevance of the tubulin-transport system in humans.

In summary, in normal monocytes, microtubules mediate intracellular transport of 25(OH)D3 to the mitochondria and the translocation of the 1,25(OH)2D3-receptor complex to the nucleus. Disruption of microtubular integrity markedly impaired 1,25(OH)2D3 synthesis by the mitochondrial 1α-hydroxylase and totally blocked the ability of monocytes to respond to 1,25(OH)2D3 which clearly indicate the physiological and/or pathophysiological relevance of the tubulin-transport system in humans.

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