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γ-Interferon-Induced Resistance to 1,25-$(OH)_2$ D_3 in Human Monocytes and Macrophages: A Mechanism for the Hypercalcemia of Various Granulomatoses*

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ABSTRACT

The hypercalcemia of various granulomatoses is caused by endogenous 1,25-dihydroxyvitamin D [1,25-$(OH)_2D_3$] overproduction by disease-activated macrophages. The inability of 1,25$(OH)_2D_3$ to suppress its synthesis in macrophages contrasts with the tight control of its production in macrophage precursors, peripheral blood monocytes (PBM). We examined whether 1,25$(OH)_2D_3$ resistance develops as PBM differentiate to macrophages or with macrophage activation. Normal human pulmonary alveolar macrophages (PAM) are less sensitive to 1,25$(OH)_2D_3$ than PBM, despite similar vitamin D receptor content; however, both PBM and PAM respond to exogenous 1,25-$(OH)_2D_3$ by inhibiting 1,25$(OH)_2D_3$ synthesis and inducing 1,25$(OH)_2D_3$ degradation through enhancement of 24-hydroxylase mRNA levels and activity. The human monocytic cell line THP-1 mimics PAM in 1,25$(OH)_2D_3$ synthesis and sensitivity to exogenous 1,25$(OH)_2D_3$. We utilized THP-1 cells to examine the response to 1,25$(OH)_2D_3$ with macrophage activation. Activation of THP-1 cells with γ-interferon (γ-IFN) enhances 1,25$(OH)_2D_3$ synthesis 30-fold, blocks 1,25-$(OH)_2D_3$ suppression of its synthesis, and reduces by 42.2% 1,25-$(OH)_2D_3$ induction of its degradation. The antagonistic effects of γ-IFN are not merely restricted to enzymatic activities. In THP-1 cells and in normal PBM, γ-IFN inhibits 1,25-$(OH)_2D_3$ induction of 24-hydroxylase mRNA levels without reducing mRNA stability, suggesting γ-IFN inhibition of 1,25$(OH)_2D_3$ transactivating function. These results explain 1,25$(OH)_2D_3$ overproduction in granulomatoses and demonstrate potent inhibition by γ-IFN of 1,25$(OH)_2D_3$ action in immune cells. (*J Clin Endocrinol Metab* **82:** 2222–2232, 1997)

THE HYPERCALCEMIA associated with sarcoidosis, tuberculosis, various granulomatoses and rheumatoid arthritis is caused by endogenous overproduction of 1,25-dihydroxyvitamin D [1,25-$(OH)_2D_3$] (1), the most active metabolite of vitamin D (2), by the disease activated macrophage.

Under normal physiological circumstances, 1,25$(OH)_2D_3$ is synthesized almost exclusively in the kidney. The renal 1α-hydroxylation of 25$(OH)D_3$ is catalyzed by a cytochrome P450-linked mixed function oxidase located in the mitochondria of the proximal tubules (3). Renal 1α-hydroxylase activity is under stringent regulation by serum levels of parathyroid hormone, calcium, phosphorus and 1,25-$(OH)_2D_3$ itself (3), so that serum 1,25$(OH)_2D_3$ levels remain within the normal range even in cases of vitamin D intoxication in which circulating 25OHD_3 levels increase 70-fold above normal (4). In contrast, in sarcoidosis and tuberculosis, macrophage 1,25$(OH)_2D_3$ synthesis correlates with 25OHD_3 levels (5) and the degree of the inflammatory response in the host (6), and 1,25$(OH)_2D_3$ overproduction occurs despite severe hypercalcemia, suppressed PTH levels, and supranormal concentrations of 1,25$(OH)_2D_3$ (7–9). These observations suggested that nonrenal 1α-hydroxylases do not respond to the modulators of the renal enzyme. In fact, studies *in vitro* using sarcoid (6, 10) or cytokine activated macrophages (11) demonstrated that the 1α-hydroxylation reaction is relatively immune to stimulation by calcium, PTH, or to feedback inhibition by 1,25$(OH)_2D_3$. The resistance of activated macrophages to 1,25$(OH)_2D_3$ inhibition of its own production contrasts markedly with the high sensitivity to 1,25$(OH)_2D_3$ of peripheral monocytes, the precursors of tissue macrophages. Similar to the renal enzyme, monocyte 1α-hydroxylase activity is profoundly suppressed by physiological concentrations of 1,25$(OH)_2D_3$ *in vitro* (12), and *in vivo* as demonstrated in peripheral monocytes from hemodialysis patients undergoing 1,25$(OH)_2D_3$ replacement therapy (13). In order to characterize the cause of this discrepancy, we examined the mechanisms involved in 1,25$(OH)_2D_3$ control of its synthesis in normal monocytes, and whether the response to the sterol in cells of the monocyte-macrophage lineage decreases as monocytes differentiate to tissue macrophages or as a result of macrophage activation.

An alternative mechanism for 1,25-$(OH)_2D_3$ to control its net production, and therefore its plasma levels in humans, is to increase its metabolic clearance rate (14) through the enhancement of 24-hydroxylase activity, the enzyme responsible for 1,25$(OH)_2D_3$ inactivation in mammals (2, 3). 1,25$(OH)_2D_3$ degradation occurs mainly in kidney and intestine (15–17), where the sterol induces the expression of 24-hydroxylase through a typical steroid-like mechanism

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(18, 19). 1,25$(OH)_2D_3$ binds its intracellular vitamin D receptor (VDR), and the 1,25$(OH)_2D_3$-VDR complex interacts with additional nuclear transcription factors, and with specific vitamin D responsive elements in the promoter region of the 24-hydroxylase gene enhancing the rate of transcription (20). A discrepancy similar to that described for 1,25$(OH)_2D_3$ control of its synthesis also exists between normal PBM and disease activated macrophages in 1,25$(OH)_2D_3$-induction of its degradation. Early studies utilizing sarcoid PAM (10, 11) or cytokine activated PAM (21), before the cloning of the human 24-hydroxylase, did not demonstrate induction of 24-hydroxylase activity even in response to concentrations of exogenous 1,25-$(OH)_2D_3$ 400 times above normal (10). Interestingly, VDR content was not reduced in activated macrophages (22). In contrast, in normal monocytes, physiological concentrations of 1,25-$(OH)_2D_3$ induce 25OHD_3 (12, 23) and 1,25-$(OH)_2D_3$ degradation (24). The actual contribution of the C24-oxidation pathway in both processes in monocytes, however, has not been evaluated. It is possible that 1,25-$(OH)_2D_3$ induction of its degradation in human monocyte-macrophages does not involve C24-hydroxylation. Alternatively, 1,25-$(OH)_2D_3$-mediated transcriptional activation of the 24-hydroxylase gene may be impaired along monocytic differentiation to macrophages or as a direct result of macrophage activation.

To identify the mechanisms mediating 1,25$(OH)_2D_3$ resistance, first, we assessed the involvement of rapid (nongenomic) and/or steroid-like (genomic) mechanisms in 1,25$(OH)_2D_3$ control of its production in PBM. Next, we examined the role of differentiation by comparing the response to 1,25$(OH)_2D_3$ in the inhibition of its synthesis as well as in the induction of catabolism between resting peripheral monocytes and pulmonary alveolar macrophages from normal adults. We present evidence that the human monocytic cell line THP-1 constitutes a proper model of tissue macrophage. Finally, we examined the effects of activation of normal monocytes and THP-1 cells with the cytokine γ-IFN on 1,25$(OH)_2D_3$ modulation of the activity of 1α- and 24- hydroxylases, and in 1,25$(OH)_2D_3$-mediated induction of 24-hydroxylase gene transcription.

We demonstrate the existence of potent antagonistic effects of γ-IFN not only on 1,25$(OH)_2D_3$ control of the activities of 1α- and 24-hydroxylases, but on 1,25$(OH)_2D_3$-induction of 24-hydroxylase mRNA levels. The latter is not the result of a decreased half life of 24-hydroxylase mRNA, suggesting that γ-IFN impairs 1,25$(OH)_2D_3$ transactivating function in normal monocytes and in THP-1 cells leading to vitamin D resistance.

Materials and Methods

Materials

25-hydroxy[26(27)-methyl ^{3}H]cholecalciferol, [specific activity (S.A.): 10–30 Ci/mmol], 1α,25-hydroxy[26,27 methyl-^{3}H]cholecalciferol (S.A.: 130–180 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). 1,25-$(OH)_2D_3$, 24,25$(OH)_2D_3$ and 1,24,25$(OH)_3D_3$ were kindly provided by Dr. Milan Uskokovic (Hoffman-La Roche Nutely, NJ) and 1,25-dihydroxy-24-oxo-vitamin D_3 by Dr. G.S. Reddy (Women and Infants Hospital, Providence, RI). Recombinant human γ-interferon (S. A.: 20–40 million IU/ml) was a generous gift from Genentech (South San Francisco, CA).

Cell culture

Peripheral blood mononuclear cells were isolated from normal volunteers using a Ficoll-Paque gradient (Pharmacia LKB Biotechnology, Piscataway, NJ) and processed as previously described (12, 23). Briefly, cells were plated in six-well plates at a concentration of 10^7 cells/well in 1 mL RPMI 1640 medium containing 1% fatty acid free bovine serum albumin (BSA). After an 18 h incubation at 37 C in humidified 95% air, 5% CO_2, nonadherent cells and medium were removed. More than 95% of these adherent cells stained positively for α-naphthyl esterase, a specific marker for cells of the monocyte-macrophage lineage (25). The adherent cell population was used in all studies.

Human alveolar macrophages were isolated from healthy adult volunteers by saline bronchoalveolar lavage using a protocol approved by the Human Study Committee at Washington University Medical Center, as previously described (26, 27). Macrophages were plated in 1 mL of Hank's balanced salt solution at a concentration of 10^6 cells per well and incubated for 1 h at 37 C to allow attachment. The medium was then replaced with 1 mL RPMI 1640 containing 1% fatty acid free BSA, and cells were incubated at 37 C in humidified 95% air, 5% CO_2 for 18 h before use.

The human monocytic cell line THP-1 (kindly provided by Dr. Beth Lee, Renal Division, Washington University Medical Center, St. Louis, MO) was grown in suspension in RPMI 1640 containing 10% fetal bovine serum (FBS) and induced to acquire a macrophage phenotype (28) by exposure to 160 nmol/L phorbol 12-myristate 13-acetate (TPA) (Sigma, St. Louis, MO) for 24 h in six-well plates, at a concentration of 2×10^6 cells/well. The medium was then exchanged for 1 mL RPMI 1640 containing 1% fatty acid free BSA and the adherent, phorbol differentiated THP-1 cells (dTHP-1 cells) were incubated at 37 C in humidified 95% air, 5% CO_2 for 18 h before use.

Time course for the effects of 1,25-$(OH)_2$ D_3 on vitamin D metabolism in normal human monocytes

Normal peripheral blood monocytes plated in RPMI 1640 containing 1% fatty acid free BSA were exposed to 0 (Control) or 0.24 nmol/L 1,25-$(OH)_2D_3$ for 0.5, 1, 2, 3, and 4 h. Medium was removed, and cells were washed once with PBS and twice with incubation medium (RPMI 1640 containing 0.1% fatty acid free BSA) to remove exogenous 1,25-$(OH)_2D_3$. The rates of conversion of tritiated 25OHD_3 to 1,25-$(OH)_2D_3$ and to metabolites more polar than 1,25-$(OH)_2D_3$ (polar metabolites) were measured as described previously (12, 23). Results were expressed as percent of control values (untreated monocytes) for both 1,25-$(OH)_2D_3$ inhibition of its synthesis and for 1,25$(OH)_2D_3$ induction of catabolic pathways. For each time point, determinations were performed in triplicate. To identify the hydroxylation pathway induced by 1,25-$(OH)_2D_3$ in normal human monocytes, polar metabolites synthesized by untreated monocytes and by monocytes exposed to 0.24 nmol/L 1,25-$(OH)_2D_3$ for 18 h were extracted, dried under nitrogen, and incubated with 500 μL of 8% sodium periodate ($NaIO_4$), 200 μL K_3PO_4, 10 mmol/L, pH 7.4, and 300 μL acetic acid 0.1 mol/L for 30 min at 0–4 C (29, 30). At the end of the incubation, 1 mL acetonitrile and 0.5 mL of 0.4 mol/L K_2HPO_4 (pH = 10.6) were added, samples were centrifuged at 2,500*g* for 15 min, and subsequently re-extracted using C-18 cartridges. To further characterize the polar metabolites, we performed normal phase high performance liquid chromatography (HPLC) on the acetonitrile fraction using 2.7% isopropanol in methylene chloride as the mobile phase and a flow rate of 2 mL/min. The retention times of 24,25$(OH)_2D_3$, 1,25-$(OH)_2D_3$, 1,24,25$(OH)_3D_3$, and 1,25$(OH)_2$-24-oxo-vitamin D_3 standards were 3.6, 10.2, 16.0, and 9.9, respectively. A 2-min methanol strip of the column followed the elution of the 1,24,25$(OH)_2D_3$ peak. HPLC fractions of tritiated metabolites eluting with nonradioactive standards and with methanol were collected and counted for tritium. Results were compared to control samples run in parallel but not subjected to $NaIO_4$ treatment.

Role of new protein synthesis in the induction of vitamin D catabolism by 1,25-$(OH)_2$ D_3

Monocytes were co-incubated with 0 or 0.24 nmol/L 1,25-$(OH)_2D_3$ and 0 or 0.1 μg/mL cycloheximide for 4 h at 37 C in RPMI 1640 containing 1% fatty acid free albumin. At the end of this incubation, cells

were washed twice with PBS and once with incubation medium. The rate of conversion of tritiated $25OHD_3$ to polar metabolites was measured in triplicate for each experimental condition, as described (12, 23). Results were expressed as percent over the rate of synthesis of polar metabolite by untreated monocytes.

Time course for 1,25-$(OH)_2$ D_3 induction of 24-hydroxylase mRNA levels in normal monocytes

Monocytes were incubated in RPMI 1640 containing 1% fatty acid free BSA and 0 or 0.24 nmol/L 1,25-$(OH)_2D_3$ for 0.5, 1, 2, 4, 8, or 18 h. Medium was then removed, and cells were washed with 2 mL PBS. Total RNA was prepared using RNAzol (Tele Test Inc., Friendswood, CA) and assayed for 24-hydroxylase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels using the ribonuclease protection assay described previously (23). Briefly, total RNA was dissolved in 4 μL of diethyl pyrocarbonate water and mixed with 26 μL hybridization buffer (80% formamide, 50 mmol/L PIPES, pH = 6.4, 400 mnol/L NaCl, 1 mmol/L EDTA) containing ^{32}P labeled riboprobes for human 24-hydroxylase and glyceraldehyde-3-phosphate dehydrogenase. After hybridization at 45 C for 16 h, samples were mixed with 150 μL ribonuclease digestion mixture containing 2 μg ribonuclease T1 in 10 mmol/L Tris-HCl, pH 5.0, 300 mmol/L NaCl, 5 mmol/L EDTA, and incubated for 15 min at 37 C. Proteinase K (50 μg) and 20 μL of 5% sodium dodecyl sulfate were then added, and the samples were incubated for 15 additional minutes. Following phenolchloroform extraction and ethanol precipitation, samples were resolved on a 5% polyacrilamide gel. Bands in the dried gel were quantified by scanning densitometry after a 48 h-exposure using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Riboprobes.. The human 24-hydroxylase template is the *Bam*HI restriction fragment, bases 1657 to 1999 of the human 24-hydroxylase complementary (c)DNA (kindly provided by Dr H. DeLuca, University of Wisconsin, Madison, WI) subcloned into Bluescript KS (Stratagen, La Jolla, CA). The template for human GAPDH was purchased from Ambion (Austin, TX). For both templates, radiolabeled antisense riboprobes were produced using RNA polymerase T7. The size of the protected fragments was 342 bp for the human 24-hydroxylase and 150 for the human GAPDH.

1,25-$(OH)_2$ D_3 synthesis and the response to 1,25-$(OH)_2$ D_3 in tissue macrophages

PAM and dTHP-1 cells were incubated in 1% fatty acid free albumin RPMI 1640 with 0, 0.24, 4.8, 12, or 24 nmol/L 1,25-$(OH)_2D_3$ for 4 or 18 h, as indicated for each experimental protocol. At the end of these incubations, cells were washed twice with PBS. The synthesis of 1,25-$(OH)_2D_3$ and of polar metabolites was measured in triplicate for each dose of 1,25$(OH)_2D_3$ tested, as described for PBM. To quantitate mRNA levels, total RNA was prepared from three individual wells of cells for each experimental condition and assayed for 24-hydroxylase and GAPDH mRNA levels using the ribonuclease protection assay described above.

Vitamin D receptor content

To assess VDR content, PBM, PAM, and dTHP-1 cells were incubated with 0.26 nmol/L [^{3}H]-1,25-$(OH)_2D_3$ for 1 h at 37 C, with (nonspecific binding) or without 125 nmol/L radioinert 1,25-$(OH)_2D_3$. Maximal specific 1,25-$(OH)_2D_3$ binding to the VDR was measured as previously described (23). Briefly, cells were rinsed with PBS containing 5 mg/ml albumin, placed on ice, and sonicated for 30 sec in 2 mL TEDK buffer. Free 1,25-$(OH)_2D_3$ was separated from the bound sterol using dextran-charcoal, and tritium was counted in the supernatant.

Effect of γ-IFN on 1,25-$(OH)_2$ D_3 synthesis in dTHP-1 cells

THP-1 cells were exposed to 160 nmol/L TPA in 10% FBS RPMI 1640 for 24 h. dTHP-1 cells were then washed twice with PBS and once with serum free RPMI 1640 containing 1% fatty acid free BSA, and incubated in RPMI 1640 containing 1% BSA and 0 or 2000 IU/mL γ-IFN for 18 h. Cells were then washed and 1,25-$(OH)_2D_3$ synthesis was measured as described for PBM. For Km and Vmax determinations, 1,25-$(OH)_2D_3$ synthesis was measured in triplicate using four different concentrations of $25OHD_3$ (from 5 to 300 nM). 1,25$(OH)_2D_3$ synthesis was measured in triplicate for every substrate concentration; Km and Vmax were obtained from a linear regression analysis of the data using the double reciprocal plot of Lineweaver-Burk.

To assess whether the 1α-hydroxylation of $25OHD_3$ in dTHP-1 cells was mediated by a cytochrome P450-linked mixed function oxidase, or by nonenzymatic oxidation of $25OHD_3$ by free radicals (generated through the activation of macrophages by γ-IFN), dTHP-1 cells were incubated with 0 or 2000 IU/mL γ-IFN for 18 h as indicated for Km and Vmax determinations. Cells were then washed, and the incubation medium was replaced by 0.1% BSA- RPMI 1640 containing 10 μmol/L ketoconazole, a cytochrome P450 inhibitor, or the free radical scavengers N, N′-diphenylethylenediamine (10 μmol/L) or ethylendiaminetetracetic acid (3 mmol/L). For each experimental condition, 1,25-$(OH)_2D_3$ synthesis was measured in triplicate.

We assessed the identity of the putative 1,25$(OH)_2D_3$ generated by THP-1 cells as follows: Nonadherent THP-1 cells were plated in eight tissue culture dishes (60 $\times$ 15 mmol/L), at a concentration of 6×10^6 cells in 3 ml 10% FBS RPMI 1640 containing 160 nmol/L TPA, and incubated at 37 C for 24 h. Medium was then removed and replaced with 3 mL fresh serum free RPMI 1640 containing 1% fatty acid free BSA. 1000 U/mL γ-IFN were then added to four of the dishes. Control (untreated) and γ-IFN-treated dishes were incubated at 37 C for 18 h. Medium was then removed and replaced with fresh RPMI 1640 containing 0.5% BSA. Substrate was 50 nmol/L radioinert $25OHD_3$ in half of the control and γ-IFN treated culture dishes and 12 nmol/L ^{3}H- 25(OH)D_3 in the remnant half. Reactions were stopped by the addition of 3 mL acetonitrile after a 4 or 6 h incubation at 37 C. To quantitate recoveries, 1000 cpm ^{3}H-1,25$(OH)_2D_3$ were then added to the culture dishes incubated with radioinert $25OHD_3$, and 100 ng radioinert 1,25$(OH)_2D_3$ to those dishes in which radioactive substrate was used. Vitamin D metabolites were then extracted from all culture dishes using C18 cartridges according to Reinhardt *et al.* (31) and further purified by straight phase HPLC using methylene chloride: isopropanol (96:4) at a flow rate of 2 mL/min. Radioinert 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ standards (100 ng) were used to identify the retention times for both metabolites in control and γ-IFN treated cultures dishes. One min fractions were collected and counted for tritium. The amount of putative ^{3}H-1,25$(OH)_2D_3$ synthesized in 4 h was measured as described (12, 23), and this rate of synthesis was used to estimate the expected concentration of putative 1,25$(OH)_2D_3$ synthesized by dTHP-1 cells incubated with radioinert substrate. In these samples, the putative 1,25$(OH)_2D_3$ fractions co-eluting with 1,25$(OH)_2D_3$ standards (retention time: 11.3 min) in the first HPLC purification (methylenechloride:isopropanol) were then subjected to two additional HPLC purifications: a straight phase using hexane:isopropanol (88:12) at a flow rate of 1.8 mL/min (retention time for 1,25$(OH)_2D_3$: 9.15 min), and a reverse phase HPLC using methanol: water (87:13) at a flow rate of 2 mL/min (retention time for 1,25$(OH)_2D_3$: 13.7 min). The putative 1,25$(OH)_2D_3$ fraction was dried under nitrogen, redissolved in 200 μL ethanol, and 50 μL were counted for tritium to quantitate recoveries. Based on the expected 1,25$(OH)_2D_3$ concentration and recoveries, 2 sets of dilutions (1:100 and 1:200) were prepared for controls and γ-IFN treated samples. These dilutions were then used to test the ability of the HPLC-purified putative 1,25$(OH)_2D_3$ to displace ^{3}H-1,25$(OH)_2D_3$ from its binding to the calf thymus receptor compared with 1,25$(OH)_2D_3$ standards (1 to 20 pg/25 μL ethanol) using the radioreceptor assay of Reinhardt *et al.* (31).

In addition, we measured 24,25$(OH)_2D_3$ synthesis induced by the putative 1,25$(OH)_2D_3$ generated endogenously by THP-1 cells from ^{3}H-$25OHD_3$ in 4 and 6 h. ^{3}H-24,25$(OH)_2D_3$ was measured using the methodology described for 1,25$(OH)_2D_3$ or polar metabolite determinations.

Effect of γ-IFN on the response to 1,25-$(OH)_2$ D_3 in dTHP-1 cells

dTHP-1 cells were incubated in RPMI 1640 containing 1% fatty acid free albumin, 0 or 4.8 nmol/L 1,25-$(OH)_2D_3$, and 0 or 2000 IU/mL γ-IFN at 37 C for 18 h. Cells were then washed, and 1,25-$(OH)_2D_3$ synthesis was measured as described for PBM and PAM. To directly assess 1,25$(OH)_2D_3$ mediated induction of its own catabolism, we measured the rate of degradation of ^{3}H-1,25-$(OH)_2D_3$ as previously described (24).

Dose response to γ-IFN inhibition of 1,25-$(OH)_2$ D_3 mediated induction of 24-hydroxylase mRNA

dTHP-1 cells were incubated with 0 or 9.6 nmol/L 1,25-$(OH)_2D_3$ and increasing concentrations of γ-IFN (from 0 to 2000 IU/ml) for 18 h in serum free RPMI 1640 containing 1% fatty acid free BSA. Total RNA was prepared and assayed for 24-hydroxylase and GAPDH mRNA levels using the ribonuclease protection assay. VDR content was measured after an 18 h exposure in THP-1 cells treated with 0, 25, 100, or 2000 IU/mL. For each dose of the cytokine, VDR measurements were performed in triplicate from 2 independent experiments.

Effect of γ-IFN on 1,25-$(OH)_2$ D_3-actions in normal human monocytes

Peripheral monocytes from normal volunteers were exposed to 0 or 0.24 nmol/L 1,25-$(OH)_2D_3$ in the presence of 0 or 2000 IU/mL γ-IFN for 18 h at 37 C in serum-free (1% BSA) RPMI 1640. Cells were then washed, and 1,25-$(OH)_2D_3$ synthesis was measured in cells incubated in RPMI 1640 containing 0.1% fatty acid free albumin as described above. Total mRNA was prepared from three individual wells of monocytes for each experimental condition and assayed for 24-hydroxylase and GAPDH mRNA levels as described. Determinations of 1α-hydroxylase activity and mRNA levels were performed in triplicate.

Effect of γ-IFN on the half life of 24-hydroxylase mRNA

dTHP-1 cells were exposed to 9.6 nmol/L 1,25-$(OH)_2D_3$, and 0 or 20 U/mL γ-IFN. After an 18 h incubation, 5 μg of the RNA polymerase II inhibitor actinomycin D were added to block further transcription. Total RNA from untreated and γ-IFN treated cells was prepared at times of 0, and 1, 2, 4, or 6 h after the addition of actinomycin D. 24-hydroxylase, and GAPDH mRNA levels were quantitated by ribonuclease protection assay. Based on the single exponential decay of the 24-hydroxylase/GAPDH ratio in untreated and γ-IFN-treated dTHP-1 cells, the half life of 24-hydroxylase mRNA was calculated as ln 2/slope of the linear regression of ln (24-hydroxylase/GAPDH mRNA ratio) *vs.* time (h).

Statistics

Data are expressed as mean ± SEM. The symbol (n =) refers to the number of independent experiments performed. Statistical analysis was performed using one tailed unpaired nonparametric Mann-Whitney Two Sample Test or one way ANOVA for multiple comparisons. Paired *t* test was employed when comparing results before and after treatment in cells from the same individual.

Results

Mechanisms mediating 1,25$(OH)_2$ D_3 regulation of vitamin D metabolism in normal peripheral blood monocytes.

Because the resistance of activated macrophages to 1,25$(OH)_2D_3$-inhibition of its own synthesis is unrelated to changes in VDR content, we first examined whether the tight control by 1,25$(OH)_2D_3$ of its own synthesis in normal monocytes involved rapid, nongenomic mechanisms instead of the most common steroid-like action mediated by the VDR.

Figure 1 depicts the time course for the effects of physiological concentrations of 1,25-$(OH)_2D_3$ (0.24 nmol/L) on vitamin D metabolism in peripheral monocytes. Both effects of the sterol, the suppression of 1,25-$(OH)_2D_3$ synthesis and the induction of catabolic pathways, measured by the rate of synthesis of polar metabolites, were only evident after 2 h. In four independent experiments, the sterol was unable to reduce 1,25-$(OH)_2D_3$ synthesis or to enhance the generation of polar metabolites by PBM in 10, 20, 40, or 60 min, even at a concentration of 1,25-$(OH)_2D_3$ as high as 0.96 nmol/L (data not shown). However, because the 1α-hydroxylase has not been purified to homogeneity or cloned, we could not confirm the actual involvement of genomic mechanisms in 1,25-$(OH)_2D_3$ suppression of its own synthesis in PBM. We therefore focused on characterizing whether 1,25-$(OH)_2D_3$ induction of its own catabolism in monocytes involved a genomic mechanism. Specifically, we examined the C24-hydroxylation pathway, the major catabolic pathway induced by 1,25$(OH)_2D_3$ through a classical steroid-like mechanism in kidney and intestine in mammals (18, 19).

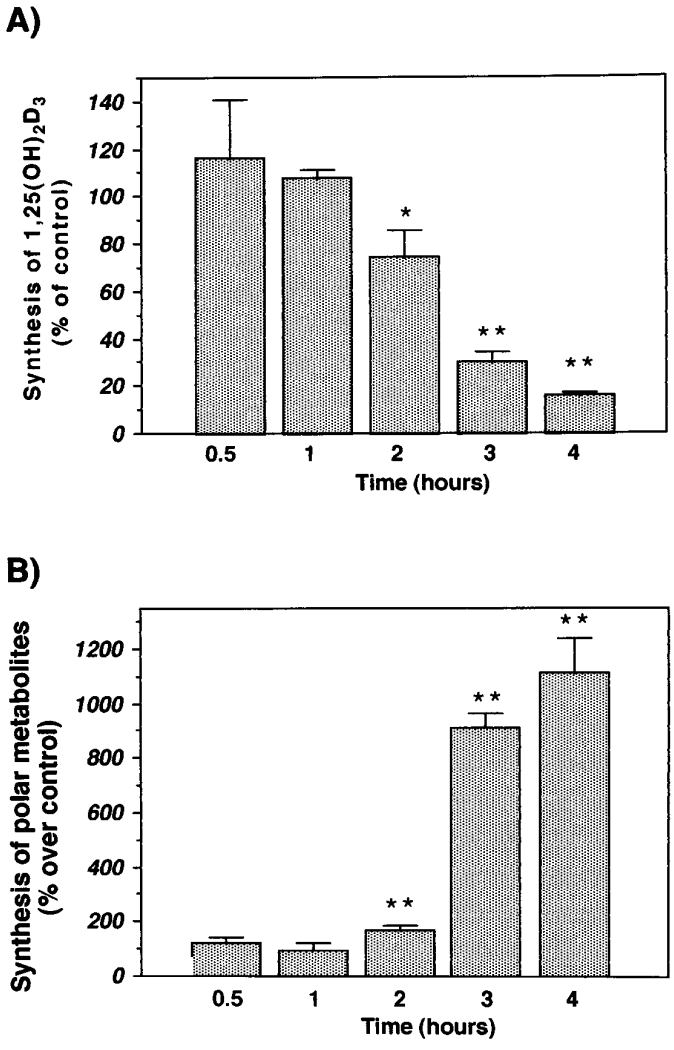

FIG. 1. Time course for the effects of 0.24 nmol/L exogenous 1,25-$(OH)_2D_3$ on A) Synthesis of 1,25-$(OH)_2D_3$ and B) Synthesis of metabolites more polar than 1,25-$(OH)_2D_3$ (polar metabolites) by PBM. Values for each time point represent the mean ± SEM of triplicate determinations and are expressed as percent of control (untreated monocytes). * and ** indicates $P \leq 0.05$ and 0.001 from controls, respectively.

Figure 2 demonstrates the results of time course studies on the induction of 24-hydroxylase mRNA levels by 0.24 nmol/L 1,25-$(OH)_2D_3$. Under basal conditions (untreated monocytes), 24-hydroxylase mRNA levels were undetectable. Incubation of PBM with 0.24 nmol/L 1,25-$(OH)_2D_3$ induced 24-hydroxylase mRNA to detectable levels after a 2-h exposure. The increase in 24-hydroxylase mRNA levels was linear with time up to 8 h and paralleled 1,25-$(OH)_2D_3$ induction of vitamin D catabolism, measured by the synthe-

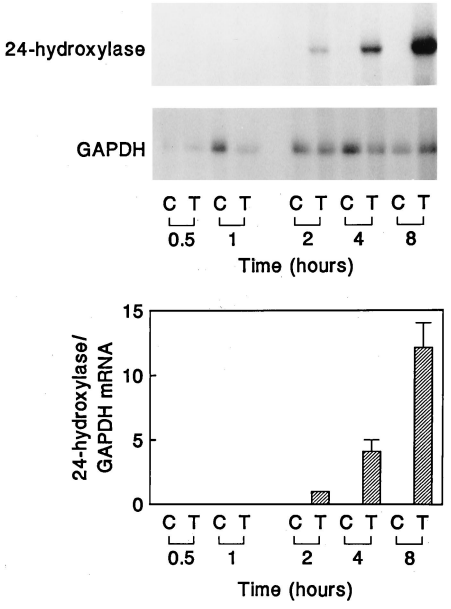

FIG. 2. Time course for 1,25-$(OH)_2D_3$-induction of 24-hydroxylase mRNA levels in peripheral blood monocytes. Total RNA from control cells (C) and cells exposed to 0.24 nmol/L 1,25-$(OH)_2D_3$ (T) for 0.5, 1, 2, 4, and 8 h was assayed for mRNA levels of 24-hydroxylase and GAPDH. A) 24-hydroxylase and GAPDH protected fragments in monocytes from the same individual; B) Densitometric analysis of the 24-hydroxylase: GAPDH mRNA ratio. Results are expressed as times over the 24-hydroxylase/GAPDH ratio at 2 h and represent the mean ± SEM of three independent experiments.

sis of polar metabolites depicted in Fig. 1. To further confirm this correlation, we examined whether the polar metabolites were 24-hydroxylated by using the classical periodate cleavage reaction (30) and HPLC elution profiles. Polar metabolites synthesized from tritiated $25OHD_3$ by untreated cells (controls) or by monocytes exposed to 0.24 nmol/L 1,25-$(OH)_2D_3$ for 18 h were subjected to 8% $NaIO_4$ for 30 min at 0–4 C and further HPLC-purified as described in Materials and Methods. $NaIO_4$ cleaves the side chain of cholecalciferols between adjacent hydroxyl groups, and because the ^3H-$25OHD_3$ used as substrate in these experiments was labeled at C26 and C27, both C24 or C26 hydroxylations would result in loss of radioactivity after periodate treatment. 25–26 dihydroxylated compounds, however, would only lose half of the tritium. Control experiments using tritiated $25OHD_3$ and 1,25-$(OH)_2D_3$ showed no degradation of these metabolites by $NaIO_4$. In addition, $NaIO_4$ treatment had no effect on the 1,25-$(OH)_2D_3$ produced by the cells, confirming its specificity of action for vitamin D metabolites containing diols. In three independent experiments, $NaIO_4$ treatment resulted in a total loss of radioactivity eluting with the polar metabolite fraction in 1,25-$(OH)_2D_3$-treated monocytes. The simultaneous detection by HPLC of tritiated metabolites coeluting with 1,24,25$(OH)_3D_3$ and 1,25$(OH)_2$-24-oxo-vitamin D standards in monocytes treated with 0.24 nmol/L 1,25-$(OH)_2D_3$ for 4 h, but not in untreated monocytes, suggested that induction of the 24-hydroxylation pathway in normal human PBM might partially account for the increase in polar metabolite synthesis by 1,25-$(OH)_2D_3$. Furthermore, 1,25-$(OH)_2D_3$ induction of vitamin D catabolism (synthesis of polar metabolites) required ongoing protein synthesis. Incubation of monocytes with 0.1 μg/mL cycloheximide for 4 h did not affect monocyte viability as measured by trypan blue exclusion. However, we found that the 5.7-fold induction of the synthesis of polar metabolites by 0.24 nmol/L 1,25-$(OH)_2D_3$ over that of untreated monocytes was totally abolished ($P < 0.001$) in monocytes exposed to the same concentration of the sterol in the presence of cycloheximide. These results demonstrate that, in normal monocytes, 1,25$(OH)_2D_3$ inhibition of its synthesis does not involve rapid mechanisms and that, similar to kidney and intestine, 1,25$(OH)_2D_3$ induces its degradation through enhancement of 24-hydroxylase mRNA levels and activity.

Vitamin D metabolism and its regulation by 1,25(OH)$_2$ D$_3$ in normal pulmonary alveolar macrophages

To assess whether the ability of 1,25$(OH)_2D_3$ to control its own synthesis and catabolism is lost as peripheral monocytes differentiate to tissue macrophages, we examined 1,25$(OH)_2D_3$ production and its regulation by exogenous 1,25$(OH)_2D_3$ in PAM obtained from healthy adult volunteers by bronchial lavage. Normal human PAM constitutively express 1α-hydroxylase activity. They convert $25OHD_3$ (S.A.: 27.7 Ci/mmol; 0.1 uCi) to 1,25-$(OH)_2D_3$ at a rate of 5.2 ± 1.4 fmol/μg DNA/h (n = 4), similar to that of PBM. Fig. 3 shows a dose response to exogenous 1,25$(OH)_2D_3$ in the control of its synthesis (*left panel*) and the induction of catabolism (*right panel*) in normal PAM. The left panel depicts that a 0.24 nmol/L dose of 1,25$(OH)_2D_3$ was ineffective in suppressing 1,25-$(OH)_2D_3$ production. A 4.8 nmol/L concentration of the sterol was required to reduce 1,25-$(OH)_2D_3$ synthesis by 48.0 ± 3.9% of control (untreated PAM). Similarly, the right panel shows that a 0.24 nmol/L dose of 1,25-$(OH)_2D_3$ did not induce the synthesis of polar metabolites. Even with concentrations of exogenous 1,25-$(OH)_2D_3$ that is 20–100 times higher than physiological (from 4.8–24.0 nmol/L), the induction of catabolic pathways was only 1.5-fold above control (2.1 ± 1.7 fmol/μg DNA/h) and did not reach statistical significance.

Table 1 shows 24-hydroxylase:GAPDH mRNA levels in 1,25$(OH)_2D_3$-treated PAM. Similar to the induction of activity of enzymes involved in catabolic pathways, an exogenous 1,25-$(OH)_2D_3$ concentration of 4.8 nmol/L and 18-h exposure to the sterol were required for PAM to increase 24-hydroxylase mRNA to detectable levels. The lower sensitivity to 1,25$(OH)_2D_3$ of PAM was not caused by a decrease in VDR, as we found no difference in maximal specific binding of tritiated 1,25-$(OH)_2D_3$ to the VDR between PBM and PAM (PBM: 0.65 ± 0.1 fmol/μg DNA, n = 5; *vs.* PAM: 0.67 ± 0.17, n = 3).

Vitamin D metabolism and its regulation by 1,25(OH)$_2$ D$_3$ in the human monocytic cell line THP-1

We examined the human monocytic leukemia cell line THP-1 as a potential model of tissue macrophage. THP-1 cells differentiate toward a macrophage-like state by exposure to 160 nmol/L TPA for 24 h (28). Vitamin D metabolism was measured in phorbol differentiated THP-1 cells (dTHP-1) after an 18 h incubation in serum-free RPMI 1640 containing

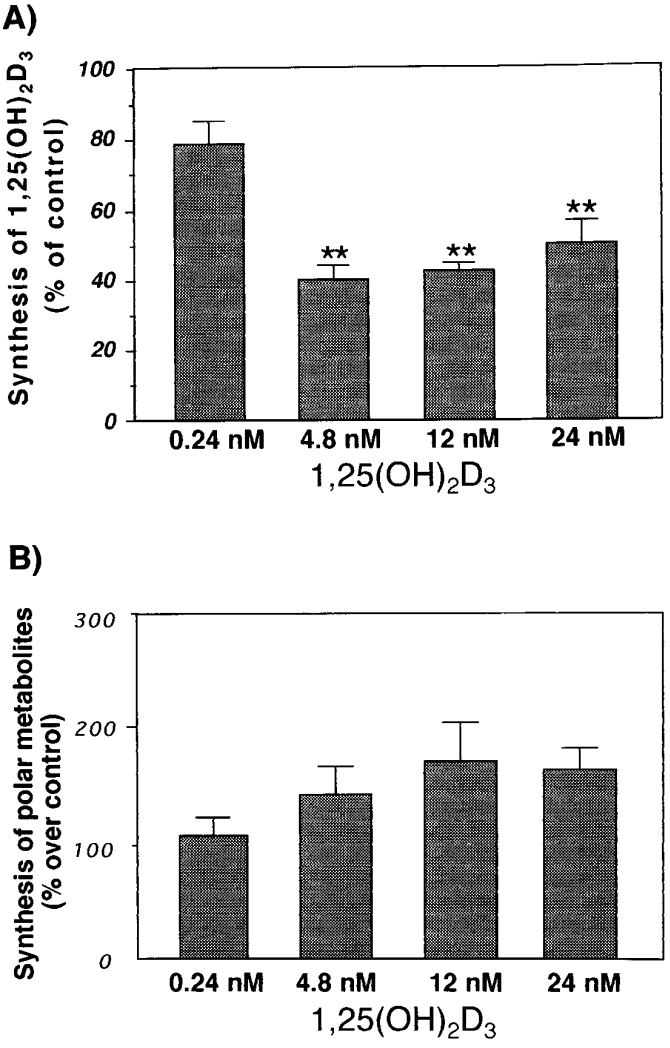

FIG. 3. Dose response for the effects of exogenous 1,25-$(OH)_2D_3$ on A) 1,25-$(OH)_2D_3$ synthesis and B) synthesis of polar metabolites by PAM. PAM were exposed to 0; 0.24; 4.8; 12, or 24 nmol/L 1,25-$(OH)_2D_3$ for 4 h. Values represent the mean ± SEM of duplicate determinations in two independent experiments. * and ** indicate $P \leq 0.05$ and 0.01 from control values, respectively.

TABLE 1. $1,25(OH)_2D_3$ induction of 24-hydroxylase mRNA levels in PBM and PAM

$1,25(OH)_2D_3$ (nmol/L)	24-Hydroxylase:GAPDH mRNA	
	PBM	PAM
0.24	0.147 ± 0.05 (7)	Undetectable
4.8	0.800 ± 0.115 (2)	0.028 ± 0.07 (3)

PBM and PAM were incubated in serum-free RPMI (1% BSA) with 0, 0.24, or 4.8 nM $1,25(OH)_2D_3$ for 18 h. Total RNA was assayed for 24-hydroxylase and GAPDH mRNA levels. Results are expressed as the mean ± SEM 24-hydroxylase:GAPDH ratio from the number of independent experiments shown in *parentheses*, performed in triplicate.

1% fatty acid free albumin. Table 2 demonstrates that dTHP-1 cells convert $25OHD_3$ to 1,25-$(OH)_2D_3$ at a rate similar to that of PAM. Also, similar to PAM, exposure of dTHP-1 cells to 2000 IU/mL of γ-IFN for 18 h produced a 30-fold increase in the Vmax of the 1α-hydroxylase. 1,25-$(OH)_2D_3$ production by resting and γ-IFN activated THP-1 cells was totally blocked by 10 μmol/L ketoconazole, a cytochrome P450 inhibitor (Table 3). Basal and γ-IFN stimulated 1,25-$(OH)_2D_3$ production by THP-1 were not affected by the presence of the free radical scavengers EDTA (3 mmol/L), or by N, N′-diphenylethylenediamine (10 μmol/L), demonstrating that conversion of $25OHD_3$ to 1,25-$(OH)_2D_3$ is the result of enzymatic oxidation of $25OHD_3$ involving a cytochrome P450-linked hydroxylation.

TABLE 2. Kinetic parameters of the 1α-hydroxylase of THP-1 cells

	Control	IFNγ
K_m	6.2 ± 1.6 (4)	8.9 ± 5.6 (2)
V_{max}	22.7 ± 5.8 (4)	684.0 ± 323.1 (2)[a]

dTHP-1 cells were incubated with 0 or 2000 IU/ml IFNγ for 18 h. 1,25-$(OH)_2D_3$ production was measured using four different substrate concentrations (from 5–300 nmol/L). K_m (nanomolar concentrations) and V_{max} [fentomoles of 1,25-$(OH)_2D_3$ per μg DNA/h) values were obtained using the double reciprocal plot of Lineweaver and Burk. Data represent the mean ± SEM from the number of independent experiments shown in *parentheses*, performed in triplicate.

[a] $P < 0.006$.

TABLE 3. The effects of cytochrome P450 inhibitors and free radical scavengers on 1,25-$(OH)_2D_3$ production by THP-1 cells

	Control	IFNγ
Basal	100 ± 10.6	1310 ± 160.7
Ketoconazole	20.1 ± 9.2[a]	2.3 ± 0.95[a]
EDTA	76.5 ± 5.5	69.7 ± 7.4
DPPD	78.4 ± 14.8	79.1 ± 8.9

dTHP-1 cells were incubated in serum-free RPMI 1640 (1% BSA) with 0 or 2000 IU/ml IFNγ for 18 h. Medium was removed, and the 1,25-$(OH)_2D_3$ produced in 1 h in the presence of the cytochrome P450 inhibitor ketoconazole (10 μmol/L) or the free radical scavengers EDTA (3 mM) or *N*,*N*′-diphenylenethylendiamine (DPPD; 10 μmol/L) was measured as described in *Methods*. Results are expressed as a percentage of basal production. The data represent the mean ± SEM of two independent experiments, performed in triplicate.

[a] $P < 0.01$ *vs.* control values.

We demonstrated that the metabolite synthesized by dTHP-1 cells under basal or γ-IFN induced states is $1,25(OH)_2D_3$ by the following criteria: 1) coelution with $1,25(OH)_2D_3$ standards in two straight phase and one reverse phase HPLC purifications: $1,25(OH)_2D_3$ production by γ-IFN treated THP-1 cells was high enough to allow the detection at a wavelength of 265 nm of a peak with the retention time of authentic $1,25(OH)_2D_3$ in the chromatograms from the three HPLC systems; 2) the ability of the endogenously produced $1,25(OH)_2D_3$ to induce 24-hydroxylase activity: 60 and 287 fmoles of $24,25(OH)_2D_3$ were generated in 4 and 6 h, respectively, by resting THP-1 cells incubated with 12 nmol/L ^3H-$25OHD_3$ alone. In γ-IFN treated cells, there was no detectable $24,25(OH)_2D_3$ despite endogenous $1,25(OH)_2D_3$ levels 5.6-fold above those of resting THP-1 cells; and 3) the affinity of the HPLC purified putative $1,25(OH)_2D_3$ for the VDR: comparison of the displacement of ^3H-$1,25(OH)_2D_3$ from the calf thymus receptor by $1,25(OH)_2D_3$ standards and different dilutions of the HPLC-purified putative $1,25(OH)_2D_3$ synthesized in 4 h rendered actual concentrations of putative $1,25(OH)_2D_3$ of 3.7 and 3.1 ng in control cells and 18.3 ng in γ-IFN treated THP-1 cells.

These values did not differ from the expected concentrations of 3.9, 2.2, and 15.3 ng estimated from the rate of conversion of ^{3}H-25OHD$_3$ to ^{3}H-1,25(OH)$_2$D$_3$ using one single HPLC purification after correction for recoveries.

We next examined whether dTHP-1 cells respond to exogenous 1,25(OH)$_2$D$_3$ like PBM or PAM. Similar to PAM, THP-1 cells did not respond to 0.24 nmol/L 1,25-(OH)$_2$D$_3$ even after an 18-h exposure. They required 4.8 nmol/L 1,25-(OH)$_2$D$_3$ to suppress 1,25-(OH)$_2$D$_3$ synthesis by 67.3% ($P < 0.05$) and to induce vitamin D catabolism 2.3-fold over controls ($P < 0.05$) in 4 h. Figure 4 shows that 1,25-(OH)$_2$D$_3$ induction of 24-hydroxylase mRNA levels also required a concentration of 1,25-(OH)$_2$D$_3$ 10 times higher than that effective in PBM. The lower sensitivity of THP-1 cells is unrelated to a reduced VDR content as the maximal specific binding of 1,25(OH)$_2$D$_3$ to the VDR in THP-1 cells was 0.60 ± 0.05 fmol of 1,25-(OH)$_2$D$_3$/ug DNA; n = 4, similar to that of PAM. These studies rendered THP-1 cells the first available model of human macrophages to study cytokine regulation of 1,25-(OH)$_2$D$_3$ action.

Effects of γ-IFN on 1,25(OH)$_2$ D$_3$ action

To assess the effects of macrophage-activation on the response to 1,25-(OH)$_2$D$_3$, dTHP-1 cells were coincubated with 9.6 nmol/L 1,25-(OH)$_2$D$_3$, a dose effective to inhibit synthesis and induce catabolism, in the absence (controls) or in the presence of 2000 IU/mL of γ-IFN, the cytokine responsible for endogenous 1,25(OH)$_2$D$_3$ overproduction in sarcoidosis and tuberculosis (32, 33), at 37 C for 18 h. γ-IFN blocked the ability of exogenous 1,25-(OH)$_2$D$_3$ to suppress 1α-hydroxylase activity and caused a 42.2% reduction of 1,25-(OH)$_2$D$_3$-mediated induction of 1,25-(OH)$_2$D$_3$ degradation [Control: 13.2 ± 2.3 fmol of 1,25-(OH)$_2$D$_3$/μg DNA/h, n = 2; γ-IFN: 7.5 ± 0.3, n = 2; $P < 0.05$].

We next examined whether the inhibitory effect of γ-IFN was limited to 1,25(OH)$_2$D$_3$ modulation of the activity of 1α- and 24-hydroxylases, or if it extended to 1,25(OH)$_2$D$_3$ induction of 24-hydroxylase gene transcription. THP-1 cells were exposed to 0 (control) or 9.6 nmol/L 1,25-(OH)$_2$D$_3$, a dose that effectively induces 24-hydroxylase mRNA, and increasing concentrations of γ-IFN (from 5–2000 IU/mL) for 18 h. Figure 5 shows that γ-IFN blocks the ability of 1,25-(OH)$_2$D$_3$ to induce 24-hydroxylase mRNA in THP-1 cells in a dose dependent manner. Five IU/mL γ-IFN decreased 24-hydroxylase mRNA levels by 50.1% with maximal inhibition (86%) achieved with concentrations of the cytokine higher than 20 IU/mL. We next measured VDR content of γ-IFN-treated dTHP-1. In two independent experiments, maximal specific binding of 1,25(OH)$_2$D$_3$ to the VDR was similar in controls (0.67 ± 0.04 fmol 1, 25(OH)$_2$D$_3$/μg DNA) and in THP-1 cells treated with 25 IU/mL of γ-IFN (0.59 ± 0.02 fmol 1, 25(OH)$_2$D$_3$/μg DNA), a dose that elicited maximal inhibition of 1,25(OH)$_2$D$_3$-mediated increase in 24-hydroxylase mRNA levels. Higher concentrations of the cytokine (100 and 2000 IU/mL) reduced 1,25(OH)$_2$D$_3$ binding to the VDR to 69.0% ± 7.1 and 69.2% ± 9.6 ($P < 0.05$) of controls, respectively. Thus, the decrease in 1,25(OH)$_2$D$_3$-VDR binding with increasing γ-IFN concentrations cannot account for the dramatic loss of responsiveness.

To assess whether the observed inhibitory effects of γ-INF on 1,25-(OH)$_2$D$_3$ actions were physiologically relevant or an artifact of the treatment with phorbol-ester to induce THP-1 cells to differentiate to a macrophage-like phenotype, we used peripheral blood monocytes from normal volunteers to examine the effects of 2000 U/mL γ-INF on 1,25-(OH)$_2$D$_3$ suppression of 1α-hydroxylase activity, and on 1,25-(OH)$_2$D$_3$-mediated induction of 24-hydroxylase mRNA. Similar to the effects of the cytokine on dTHP-1 cells, 2000 IU/ml γ-IFN blocked the ability of 1,25-(OH)$_2$D$_3$ (0.24 nmol/L) to inhibit 1α-hydroxylase activity. In addition, Fig. 6 shows the effects of 18 h exposure to a dose of 2000 IU/mL of γ-IFN on the induction of 24-hydroxylase mRNA by 0.24 nmol/L 1,25(OH)$_2$D$_3$ in PBM from 4 normal adults. 24-hydroxylase mRNA levels were undetectable in control cells (untreated monocytes). Treatment with γ-IFN reduced by 79.4% ± 9.5

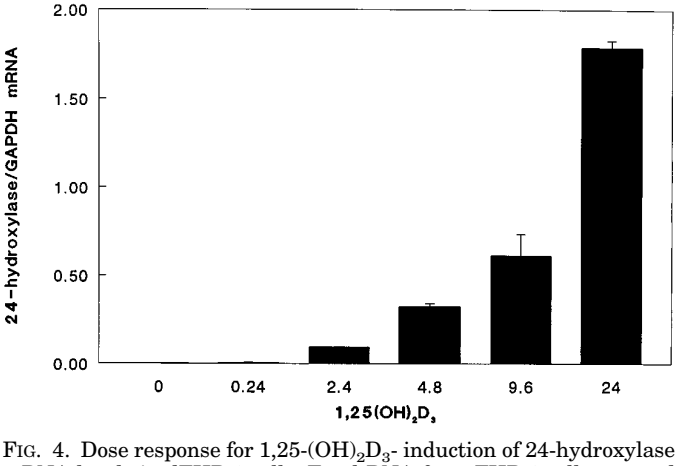

FIG. 4. Dose response for 1,25-(OH)$_2$D$_3$- induction of 24-hydroxylase mRNA levels in dTHP-1 cells. Total RNA from THP-1 cells exposed to 0, 0.24, 2.4, 4.8, 9.6 or 24, nmol/L 1,25-(OH)$_2$D$_3$ for 18 h was assayed for mRNA levels of 24-hydroxylase and GAPDH. The data represent the mean ± SEM of the 24-hydroxylase/GAPDH ratio from duplicate determinations.

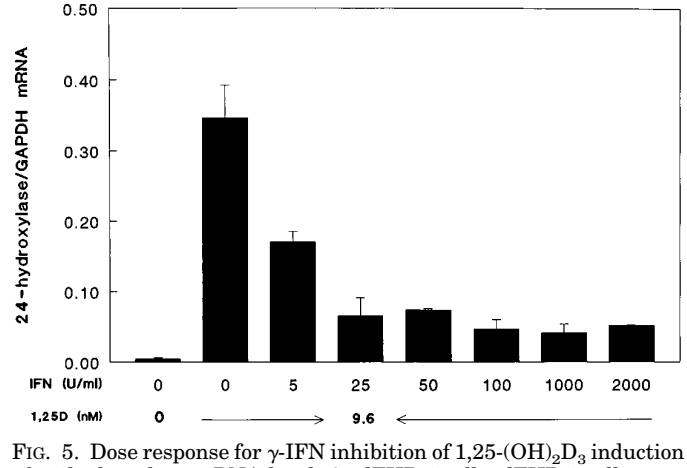

FIG. 5. Dose response for γ-IFN inhibition of 1,25-(OH)$_2$D$_3$ induction of 24-hydroxylase mRNA levels in dTHP-1 cells. dTHP-1 cells were incubated with 0 or 9.6 nmol/L 1,25-(OH)$_2$D$_3$ (1, 25(OH)$_2$D$_3$) and increasing concentrations of γ-IFN (IFN) from 0–2000 IU/ml for 18 h. Total RNA was assayed for 24-hydroxylase and GAPDH mRNA levels. Results represent the mean ± SEM of the 24-hydroxylase/GAPDH mRNA ratio from duplicate determinations.

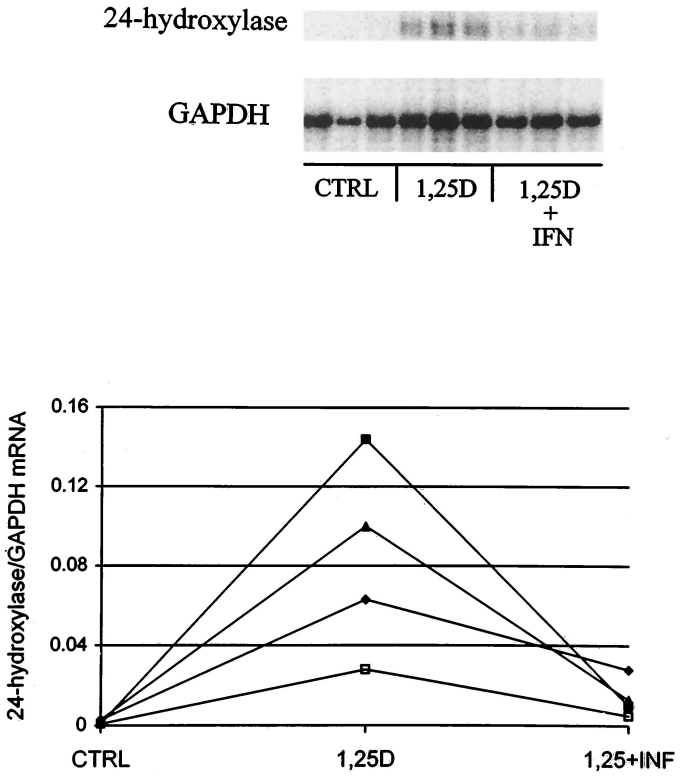

FIG. 6. Effect of γ-IFN on $1,25(OH)_2D_3$ induction of 24-hydroxylase mRNA levels in PBM. Total RNA from untreated PBM (Ctrl) or from cells exposed to 0.24 nmol/L $1,25(OH)_2D_3$ alone (1, 25D) or with 2000 IU/mL γ-IFN (1, 25D + γ-IFN) was assayed for mRNA levels of 24-hydroxylase and GAPDH. *Upper panel:* 24-hydroxylase and GAPDH protected fragments in monocytes from the same individual. *Lower panel:* Densitometric analysis of the 24-hydroxylase:GAPDH ratio in PBM from 4 normal volunteers. Values for each individual studied represent the mean of triplicate determinations.

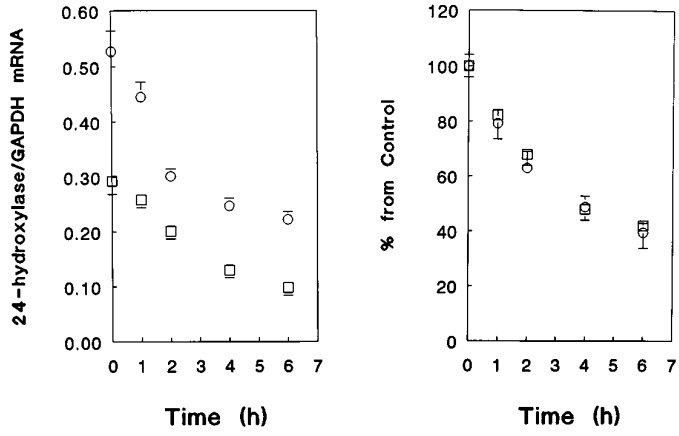

FIG. 7. Effect of γ-IFN on the half life of 24-hydroxylase mRNA. dTHP-1 cells were exposed to 9.6 nmol/L $1,25-(OH)_2D_3$ and 0 or 20 U/mL γ-IFN for 18 h. 5 μg of the RNA polymerase inhibitor actinomycin D were then added to block further transcription. Total mRNA was collected at time 0 and at 1,2,4, and 6 h after the addition of actinomycin D and assayed for 24-hydroxylase and GAPDH mRNA levels. *Panel A* depicts the decay in 24-hydroxylase/GAPDH ratio in untreated (○) and γ-IFN treated (□) dTHP-1 cells. The data represent the mean ± SEM of triplicate determinations from one representative experiment. *Panel B* shows the results of triplicate determinations from two independent experiments. The data represents mean ± SEM of 24-hydroxylase/GAPDH mRNA levels expressed as percent from the ratio at time 0 after the addition of actinomycin D.

the 24-hydroxylase:GAPDH mRNA ratio induced by $1,25(OH)_2D_3$.

A potential post-transcriptional mechanism for γ-IFN to reduce $1,25(OH)_2D_3$ induction of 24-hydroxylase mRNA levels would be to decrease the stability of 24-hydroxylase mRNA. To test this possibility, we measured the half life of 24-hydroxylase mRNA in untreated and γ-IFN treated THP-1 cells. Fig. 7 (*left panel*) depicts the single exponential decays of 24-hydroxylase mRNA levels with time after blocking further transcription with the RNAse polymerase II inhibitor actinomycin D in γ-IFN treated and untreated THP-1 cells. In two independent experiments, the estimated half life of 24-hydroxylase mRNA was 4.5 ± 0.15 h in control cells and 4.9 ± 1.3 h in γ-IFN treated dTHP-1 cells. Clearly, γ-IFN does not reduce 24-hydroxylase mRNA stability, as better illustrated in Fig. 7 (*right panel*), when the 24-hydroxylase/GAPDH mRNA ratio is plotted as percent of time 0. This suggests an antagonistic effect of the cytokine on $1,25(OH)_2D_3$ induction of 24-hydroxylase gene transcription.

Discussion

We have identified the mechanism responsible for the resistance of disease-activated macrophages to $1,25(OH)_2D_3$, which causes supranormal serum $1,25(OH)_2D_3$ levels and hypercalcemia in various granulomatoses. The demonstration that the VDR content does not change with macrophage activation (22) suggests that $1,25-(OH)_2D_3$ inhibition of its own synthesis in cells of the monocyte-macrophage lineage may involve rapid, nongenomic mechanisms similar to the 1α-hydroxylase of kidney cell cultures (34). Time course experiments in normal PBM ruled out the involvement of rapid, nongenomic actions in $1,25-(OH)_2D_3$ control of monocytic 1α-hydroxylase. $1,25-(OH)_2D_3$ suppression of its own synthesis required at least a 2-h exposure to physiological concentrations of the sterol. Even with concentrations of $1,25-(OH)_2D_3$ ten times above the normal range, no feedback inhibition of monocyte $1,25-(OH)_2D_3$ production was detected within the first hour. Although conclusive evidence of a genomic control by the sterol of its own synthesis awaits the cloning of the 1α-hydroxylase, the recent demonstration of enhanced 1α-hydroxylase activity and high serum $1,25-(OH)_2D_3$ levels in the VDR knockout mouse (35) supports our finding that $1,25-(OH)_2D_3$ suppression of its own synthesis may be a VDR mediated process.

The striking difference in the sensitivity to $1,25(OH)_2D_3$ inhibition of 1α-hydroxylase activity between peripheral blood monocytes, the precursors of tissue macrophages, and activated macrophages raises the possibility that the cellular signaling pathways required by the sterol are lost either in the differentiation of peripheral monocytes to tissue macrophages or as a result of macrophage activation. To evaluate the contribution of monocyte differentiation in macrophage-resistance to $1,25(OH)_2D_3$, we examined $1,25(OH)_2D_3$ control of its synthesis in PAM obtained by bronchial lavage from healthy adult volunteers. PAM constitutively express 1α-hydroxylase activity, and the rate of $1,25(OH)_2D_3$ production is similar to that of PBM. However, a concentration of

$1,25(OH)_2D_3$ 20 times higher than that effective in PBM was required to reduce $1,25(OH)_2D_3$ synthesis by 50%. Thus, $1,25\text{-}(OH)_2D_3$ is capable of suppressing its synthesis in normal PAM with a lower potency. This contrasts markedly with the lack of feedback inhibition by the sterol of macrophage 1α-hydroxylase of human PAM activated *in vivo* by underlying diseases such as sarcoidosis (7), tuberculosis (8), various granulomatoses (1), and rheumatoid arthritis(9), or *in vitro* after exposure of normal PAM to γ-IFN (10) or TNFα (21). This led us to hypothesize cytokine-induced resistance to $1,25(OH)_2D_3$ in the control of its synthesis. To test this hypothesis, we overcame difficulties in obtaining human macrophages by examining the human monocytic cell line THP-1 as a potential model of tissue macrophage. THP-1 cells were chosen because of their ability to be induced to differentiate toward a macrophage-like state by exposure to phorbol esters (28). Phorbol-differentiated THP-1 cells mimic more closely monocyte derived macrophages in the expression of oncogenes and membrane proteins than their more widely used counterparts, HL-60 or U937 cells (36). We demonstrate that phorbol-differentiated THP-1 cells mimic normal PAM in the constitutive expression of 1α-hydroxylase activity and in the 30 fold-increase in $1,25(OH)_2D_3$ production in response to γ-IFN. The metabolite synthesized by resting and γ-IFN activated THP-1 cells is authentic $1,25(OH)_2D_3$ based on its chromatographic properties, the affinity for the calf thymus VDR, and the ability to induce 24-hydroxylase activity. Basal and γ-IFN induced $1,25(OH)_2D_3$ synthesis in THP-1 cells were totally blocked by the cytochrome P450 inhibitor ketoconazole, and unaffected by the free radical scavengers ethylenediamine tetracetic acid or diparaphenylenediamine. This suggests that, similar to the renal (2, 3), PBM (12), and sarcoid PAM 1α-hydroxylases (11), the conversion of $25OHD_3$ to $1,25(OH)_2D_3$ in THP-1 cells is an enzymatic cytochrome P450-linked hydroxylation. The sensitivity of THP-1 cells to suppress $1,25(OH)_2D_3$ synthesis in response to exogenous $1,25(OH)_2D_3$ is also similar to that of PAM and lower than that of PBM. Thus, THP-1 cells provide a proper model of pulmonary alveolar macrophages. The demonstration that a concentration of $1,25(OH)_2D_3$, effective to suppress 1α-hydroxylase activity in resting THP-1 cells, had no effect in the presence of γ-IFN supports the existence of cytokine-induced resistance to $1,25\text{-}(OH)_2D_3$ in the feedback inhibition of macrophage 1α-hydroxylase.

Part of the decrease in $1,25\text{-}(OH)_2D_3$ production by exogenous $1,25\text{-}(OH)_2D_3$ may result from the well known genomic action of $1,25\text{-}(OH)_2D_3$, the induction of its own degradation (15–19). In nearly all target tissues, $1,25\text{-}(OH)_2D_3$ induces 24-hydroxylase, the key enzyme in mammalian vitamin D catabolism (15–17). Previous reports from our laboratory in normal monocytes have shown that physiological concentrations of exogenous $1,25\text{-}(OH)_2D_3$ induce vitamin D catabolism (12, 23) and $1,25\text{-}(OH)_2D_3$ degradation (24); however, the contribution of the C24-hydroxylation pathway in both processes has not been evaluated. The present studies demonstrate that $1,25\text{-}(OH)_2D_3$ induction of vitamin D catabolism also requires a 2-h exposure to the sterol and ongoing protein synthesis, suggesting the involvement of a genomic mechanism. The time course for $1,25\text{-}(OH)_2D_3$ induction of 24-hydroxylase mRNA levels paralleled the increase in synthesis of 24-hydroxylated polar metabolites, suggesting that, similar to kidney (15, 16) and intestine (17), in normal PBM $1,25\text{-}(OH)_2D_3$ induces $25OHD_3$ catabolism and its own degradation through enhancement of 24-hydroxylase gene transcription. In contrast, cytokine-activated macrophages exhibit no induction of 24-hydroxylase activity by either the excessive $1,25\text{-}(OH)_2D_3$ levels endogenously generated (11, 21) or in response to concentrations of exogenous $1,25\text{-}(OH)_2D_3$ 400 times above the normal range (10). Perhaps, cytokines also impair the genomic actions of $1,25\text{-}(OH)_2D_3$, and we utilized $1,25\text{-}(OH)_2D_3$-induction of 24-hydroxylase gene transcription to further characterize the mechanisms involved.

Studies in PAM and THP-1 cells demonstrated that, similar to the response to the sterol in the control of its synthesis, a concentration of $1,25\text{-}(OH)_2D_3$ 20 times higher than that effective in normal PBM was required to induce the generation of polar metabolites and 24-hydroxylase mRNA levels. The lower potency of $1,25\text{-}(OH)_2D3$ in both cell types was not attributable to a decrease in VDR content, as the maximal $1,25\text{-}(OH)_2D_3$-VDR specific binding was similar to that observed in PBM. A similar functional block of vitamin D-dependent gene regulation by $1,25\text{-}(OH)_2D_3$ has been described in human B lymphocytes (37) despite the expression of VDR mRNA and protein suggesting that factors other than VDR content determine the magnitude of the response to the sterol.

A role for the state of differentiation rather than VDR levels in the response to exogenous $1,25\text{-}(OH)_2D_3$ has been reported for the modulation of the expression of the osteocalcin (38) and 24-hydroxylase genes (39) in rat osteoblasts, the calcium binding protein in cells of the crypt and the villus of the intestine (40), and for the induction of 24-hydroxylase in HT-29 human colon cancer cells (41) and murine keratinocytes (42). In our studies, the difference in $1,25(OH)_2D_3$ induction of 24-hydroxylase mRNA levels between peripheral monocytes and pulmonary alveolar macrophages supports the findings that the transcriptional and/or translational steps may be more sensitive in less differentiated cells than in more highly differentiated (mature) ones (38–42).

We utilized phorbol-differentiated THP-1 cells to assess the effect of activation of human macrophages with the cytokine γ-IFN, responsible for $1,25\text{-}(OH)_2D_3$ overproduction by PAM in sarcoidosis and tuberculosis (32, 33), on the genomic response to $1,25\text{-}(OH)_2D_3$. γ-IFN markedly reduced $1,25\text{-}(OH)_2D_3$-mediated induction of its own degradation. The antagonistic effects of γ-IFN were not limited to $1,25\text{-}(OH)_2D_3$ regulation of the activity of 24-hydroxylase but extended to $1,25\text{-}(OH)_2D_3$ induction of 24-hydroxylase mRNA levels. In THP-1 cells, γ-IFN decreases the $1,25\text{-}(OH)_2D_3$ induction of 24-hydroxylase mRNA levels in a concentration dependent manner with a 50% reduction at a concentration of the cytokine as low as 5 U/mL and maximal suppression for concentrations above 20 IU/mL. Although doses higher than 100 IU/mL caused a mild reduction in maximal specific binding of $1,25(OH)_2D_3$ to the VDR, it is apparent that this is not the mechanism responsible for the resistance to $1,25(OH)_2D_3$. A concentration of γ-IFN of 25 IU/mL exerts maximal inhibition of $1,25(OH)_2D_3$ action without affecting $1,25(OH)_2D_3$ binding to the VDR. The re-

duction in 24-hydroxylase mRNA levels was not caused by a post-tanscriptional effect of the cytokine on the half-life of mRNAs. An alternative explanation for the reduced 24-hydroxylase mRNA levels is that γ-IFN alters the processing of the 24-hydroxylase pre-mRNA to mature mRNA; however, this is a not a common mechanism for the control of gene expression by steroid hormones. Thus, γ-IFN may directly impair 1,25-$(OH)_2D_3$ transactivating function.

This antagonism between γ-IFN and 1,25-$(OH)_2D_3$ is physiologically relevant as γ-IFN also impairs 1,25-$(OH)_2D_3$ induction of 24-hydroxylase mRNA levels in normal human monocytes.

24-hydroxylase is expressed in almost every vitamin D-responsive tissue, which raises the possibility that the inhibitory effects of γ-IFN on 1,25-$(OH)_2D_3$ induction of 24-hydroxylase may not be restricted to monocytes and macrophages. γ-IFN antagonism might extend to renal (15, 16) and intestinal (17) 24-hydroxylases, the major contributors to systemic 1,25-$(OH)_2D_3$ inactivation. This notion is supported by the demonstration that, in patients with sarcoidosis, despite the supranormal serum concentrations of $1,25(OH)_2D_3$, there is no increase in the metabolic clearance rate of 1,25-$(OH)_2D_3$ (43).

The presence of classical vitamin D responsive elements in the promoter region of the human 24-hydroxylase (44) suggests that the antagonistic effects of γ-IFN may extend to other vitamin D-regulated genes. In fact, γ-IFN and TNFα inhibit 1,25-$(OH)_2D_3$-induced osteocalcin gene transcription in rat osteoablasts (45, 46). Both cytokines also induce the expression of the transcription factor AP-1, and it was proposed that cytokine inhibition of $1,25(OH)_2D_3$-induced expression of osteocalcin was the result of the presence of an AP-1 DNA-binding site overlapping the VDR binding domain in the promoter region of the rat osteocalcin gene (38, 47). This, however, cannot be the mechanism mediating the inhibitory effects of γ-IFN on $1,25(OH)_2D_3$-induction of 24-hydroxylase gene transcription. Although there is an AP-1 binding site in the human 24-hydroxylase promoter (44), the induction of AP-1 activity with phorbol esters stimulates rather than inhibits 1,25-$(OH)_2D_3$ transactivating function in kidney (18) and intestinal (19, 48) epithelial cells. Further studies are necessary to assess whether γ-IFN impairs the cytoplasmic to nuclear translocation of the $1,25(OH)_2D_3$-VDR complex, the interaction of the complex with the DNA, or steps downstream in the assembly or the preinitiation complex controlling the rate of 24-hydroxylase gene transcription.

Recent studies of the mechanisms for the inhibition by TNFα of $1,25(OH)_2D_3$ modulation of osteocalcin gene expression demonstrated that the cytokine induces an intranuclear repressor that decreases 1,25-$(OH)_2D_3$-stimulated retinoid × receptor-VDR binding to the vitamin D responsive element of the rat osteocalcin gene. The nucleotide sequence involved is different from the AP-1 binding domain (49, 50). There has been no further characterization of the mechanisms mediating the inhibitory effect of γ-IFN on 1,25-$(OH)_2D_3$-induction of the rat osteocalcin gene, except for the demonstration that, unlike TNFα, the effects of γ-IFN require ongoing protein synthesis (45).

In summary, the studies presented here illustrate that, in cells of the monocyte macrophage lineage, 1,25-$(OH)_2D_3$, suppression of its synthesis is not mediated by rapid, nongenomic mechanisms and that the sterol induces its catabolism by enhancing 24-hydroxylase mRNA levels and activity. The resistance of activated PAM to $1,25(OH)_2D_3$ in the control of its own production cannot be accounted for by the reduced sensitivity to the sterol as monocytes differentiate to macrophages, but results from potent antagonistic effects of the cytokine γ-IFN on 1,25-$(OH)_2D_3$ action. These findings could explain the excessive 1,25-$(OH)_2D_3$ production induced by γ-IFN in PAM from patients with sarcoidosis and tuberculosis, and they demonstrate the existence of interactions at the level of gene transcription between the cytokine γ-IFN and the steroid hormone $1,25(OH)_2D_3$ in immune cells in humans. Because macrophages in different tissues display variable phenotypes (51, 52), assessment of a role for γ-IFN-1,25-$(OH)_2D_3$ antagonism in the hypercalcemia of other inflamatory conditions awaits evaluation of cytokine-1,25-$(OH)_2D_3$ interactions in macrophages from tissues involved in those disorders. Since both γ-IFN and $1,25(OH)_2D_3$ are potent modulators of cell growth, differentiation, and immune function (53–57), the observed antagonism may have pathophysiological repercussions in inflammatory processes beyond those causing abnormal calcium homeostasis.

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