

Reduced 5-Lipoxygenase Metabolism of Arachidonic Acid in Macrophages from 1,25-dihydroxyvitamin D₃-Deficient Rats

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The peripheral blood monocyte (PBM) migrates into tissues and differentiates into mature tissue macrophages. Previous investigations from our laboratory have demonstrated that PBM have reduced 5-lipoxygenase (5-LO) metabolism of arachidonic acid (AA) and 5-LO activating protein (FLAP) expression as compared to differentiated alveolar macrophages (AM). Moreover, PBM differentiated with 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) displayed increased leukotriene synthesis and a parallel increase in FLAP expression. In the present study, we sought to examine the physiological role of 1,25-(OH)₂D₃ in the regulation of eicosanoid metabolism in terminally differentiated alveolar and peritoneal macrophages (PM), utilizing a well characterized rat model of vitamin D₃-deficiency. AM from vitamin D₃-deficient rats demonstrated reduced 5-LO metabolism of AA and a parallel reduction in FLAP expression compared to control rats. Similarly, PM from vitamin D₃-deficient rats demonstrated reduced 5-LO metabolism of AA. The effect of vitamin D₃ was specific for the 5-LO pathway, not affecting total release of AA or its metabolism via 12-lipoxygenase or cyclooxygenase

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(COX) pathways in macrophages. Furthermore, it did not affect COX protein expression in macrophages or type II alveolar epithelial cells. In control animals, 1,25-(OH)₂D₃ concentrations were greater in bronchoalveolar lavage fluid (2.6-fold) and peritoneal lavage fluid (1.6-fold) than in serum, which may account for the greater FLAP expression in AM and PM than in PBM. These observations suggest that 1,25-(OH)₂D₃ plays a physiological role in upregulating the 5-LO pathway in tissue macrophages in vivo.

Keywords: Eicosanoids; leukotrienes; prostaglandins; vitamin D₃; alveolar; peritoneal

Introduction

Macrophages provide first line protection against exogenous toxins and invading microorganisms. All tissue macrophages arise by emigration from the bloodstream and subsequent differentiation of a bone marrow-derived precursor, the peripheral blood monocyte (PBM). Differentiation of various cell types is promoted by 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃).^{4,21} In particular, 1,25-(OH)₂D₃ has been shown to differentiate PBM to a more mature macrophage phenotype, as judged by the expression of maturation-associated antigens, and release of tumor necrosis factor and interleukin-6.¹⁶

The ability of macrophages to play an important role in host defense mechanisms is related, in part, to their capacity to generate various mediators, which include eicosanoids. Leukotrienes (LTs) are potent mediators of inflammation derived from the 5-lipoxygenase (5-LO) pathway of arachidonic acid (AA) metabolism which have been implicated in a wide range of immune and inflammatory diseases, including asthma, rheumatoid arthritis, inflammatory bowel disease and psoriasis.^{10,18} In most resting cells, the enzyme 5-LO resides predominantly in the soluble subcellular fraction.^{15,27} Upon cell activation, 5-LO interacts with 5-LO activating protein (FLAP), a particulate protein²² which has been shown to be necessary for LT synthesis.⁸ Ordinarily, PBM metabolize AA to only small amounts of LTs, favoring metabolism via the prostaglandin H synthase or cyclooxygenase (COX) pathway to prostaglandins (PGs) instead.¹ We have recently shown that this lesser ability to synthesize LTs is due primarily to their reduced expression of FLAP compared to AM.⁷ Interestingly, incubation of PBM with the differentiating agent 1,25-(OH)₂D₃ resulted in parallel increases in 5-LO metabolism of endogenous AA as well as in FLAP expression.⁵

In view of the capacity of 1,25-(OH)₂D₃ to upregulate LT synthesis in vitro, we wished to determine whether 1,25-(OH)₂D₃ plays a physiological role in the regulation of the 5-LO pathway in macrophages in vivo. This

question was addressed by utilizing a well-characterized rat model of vitamin D₃-deficiency. We hypothesized that macrophages from vitamin D₃-deficient rats (D-) would demonstrate reduced 5-LO metabolism of AA and FLAP expression as compared to normal rats (D+). Both 5-LO and COX pathways, along with the expression of 5-LO, FLAP and COX proteins, were therefore compared in two terminally differentiated macrophage populations, AM and peritoneal macrophages (PM). For purposes of comparison, a non-macrophage cell, the type II alveolar epithelial cell (AEC) which lines the pulmonary alveolar space, was studied. This cell metabolizes AA largely via the COX pathway lacking appreciable levels of 5-LO or FLAP proteins.

Materials and Methods

D- and D+ rats

Animal studies were performed after approval from the Unit for Laboratory Animal Medicine at the University of Michigan. Sprague-Dawley rats specially bred from D- mothers (Harlan, Madison, WI) were housed in pathogen-free conditions under non-fluorescent lighting. The rats were rendered D- by feeding for 9 weeks with a vitamin D₃-free diet which was supplemented with 2.5% Ca²⁺ and 1.5% PO₄ (Teklad, Madison, WI, lot # 86029) to maintain serum Ca²⁺ levels within normal limits, as described.³² D+ rats were obtained from the same source and housed under identical conditions, but the above diet was supplemented daily with 2 IU vitamin D₃.³² A separate group of normal pathogen-free Sprague-Dawley rats was used to determine the 1,25-(OH)₂D₃ levels in alveolar and peritoneal lining fluid. These animals were of the same strain, sex, size, and housed under identical pathogen-free conditions as the D+ and D- animals.

Isolation and Culture of AM and PM

Paired animals from D- and D+ groups were studied in parallel. Rat AM and PM were harvested by bronchoalveolar lavage (BAL) and peritoneal lavage, respectively, as described.⁶ Lavage buffer consisted of 150 mM NaCl, 2.7 mM EDTA, 20 mM HEPES, 5.5 mM dextrose, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 mg/ml amphotericin B. Lavaged cells (BAL > 94% and peritoneal lavage > 81% macrophages) were either studied fresh or were adhered (> 98% macrophages) and cultured in medium 199 (M199) as described.²⁶

Isolation and Culture of Rat Type II AEC

Type II AEC were isolated by a standard technique.⁹ Cells were suspended in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island,

NY) containing 10% fetal calf serum (FCS) with 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 mg/ml amphotericin B at 1.5×10^6 cells/ml and 2 mls of cell suspension added to 35 mm culture dishes (Falcon). Cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C. The day of isolation and plating was designated culture day 0. By day 2, cells form a confluent monolayer of which ~94% are epithelial cells as judged by cytokeratin staining.¹⁹

1,25-(OH)₂D₃ Assay

Serum and lavage fluid samples were assayed for 1,25-(OH)₂D₃ using the 1,25-(OH)₂D ³H Radio Receptor Assay Kit (INCSTAR Corporation, Stillwater, MN) as described.¹³ Briefly, the assay involves a preliminary extraction and subsequent purification of vitamin D metabolites from serum or fluid using a C₁₈ cartridge. Quantitation is achieved via a non-equilibrium competitive protein binding assay using a 1,25-(OH)₂D ³H tracer and a thymus receptor that is specific for 1,25-(OH)₂D. Dextran-coated charcoal is then incubated with the sample-receptor cocktail, binds to the unbound hormone, and is pelleted by centrifugation. The supernatant, which contains the thymus receptor-bound hormone, is decanted into a scintillation vial and counted. Values are corrected for extraction efficiency using a recovery factor and final concentration of 1,25-(OH)₂D₃ in the sample is expressed as pg/ml.

Analysis of AA metabolism in Intact Cells

Cell lipids were prelabeled by incubation with 1 µCi [³H]AA (sp. act 60-100 Ci/mmol; Dupont-New England Nuclear, Boston MA) in M199 containing 10% FCS during overnight culture. Cells were washed and the maximal capacity for eicosanoid synthesis was determined by incubation in M199 for 30 min with ionophore A23187 (Calbiochem, La Jolla, CA), diluted to a final concentration of 1 µM in DMSO (final concentration 0.5%). Medium from cultures was extracted using C₁₈ Sep-Paks (Waters, Milford, CA), and eicosanoids were separated by reverse-phase HPLC, identified by co-elution with authentic standards, and quantitated by scintillation counting of fractions.¹ In selected experiments 5-LO metabolic capacity of unlabeled A23187-stimulated cells was estimated by enzyme immunoassay (EIA) (Cayman, Ann Arbor, MI) of the cellular supernatants for LTB₄, the major 5-LO product of rat AM and PM.²⁶

Cell Lysis and Subcellular Fractionation

Fresh or cultured cells were suspended in homogenization buffer (50 mM potassium phosphate, 100 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSE, and 60 µg/ml soybean trypsin inhibitor, pH 7.1). They

were disrupted by sonication on iced ethanol using a model 250 Sonifier (Branson Ultrasonics Corp., Danbury, CT) at power level 1 and 20% duty cycle for 1.5 min, achieving > 98% lysis. Lysates then underwent centrifugation at 100,000 × g for 60 min to obtain "soluble" (supernatant) and "particulate" (pellet) fractions. The particulate fraction was then rinsed twice and resuspended in homogenization buffer by sonication (power level 1, 100% duty cycle, 10 s). Total protein content of subcellular fractions was determined using a microtiter plate modification of the Bradford method (Pierce, Rockford IL) with BSA as standard.

Immunoblot Analysis of Total Cellular 5-LO, FLAP, and COX Proteins

Steady-state protein levels of 5-LO, FLAP, and the two isoforms of COX, COX-1 and COX-2, were determined by immunoblot analysis using a modification of methods described previously.⁶ Briefly, equal amounts of protein (5–20 μg) were separated by SDS-PAGE on 10% (5-LO, COX-1 and COX-2) and 15% (FLAP) gels, by the method of Laemmli.¹⁷ High and low molecular weight rainbow markers (Amersham, Arlington, IL) were also loaded on each gel. After overnight transfer to nitrocellulose membranes (Bio-rad Laboratories, Richmond, CA), blots were blocked by incubating for 1 h with 10% non-fat dried milk in Tris buffered saline (TBS), washed in TBS containing 0.1% Tween 20 (TBS-T), and incubated at room temperature for 1 h with rabbit polyclonal primary antibodies raised against the following: human leukocyte 5-LO (1:3000 dilution); amino acid residues 41–52 of the human FLAP sequence (1:5000 dilution) (both kindly provided by Dr. J. Evans, Merck Frosst, Dorval, Canada); sheep seminal vesicle COX-1 (1:5000 dilution) (both kindly provided by Dr. W. Smith, Michigan State University, E. Lansing, MI), and a 17-amino acid peptide derived from the murine COX-2 sequence not present in COX-1 (1:300 dilution) (kindly provided by Dr. D. DeWitt, Michigan State University). After washing, blots were incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) at a dilution of 1:5000 (5-LO, FLAP and COX-2), or 1:10,000 (COX-1) in TBS-T. Membranes were then washed and developed using the ECL chemiluminescent western blotting system (Amersham). Luminescent bands were quantitated by video densitometry using software from Scion Corp (Frederick, MD).

Data Analysis

Where indicated, data were expressed as the mean ± SEM. Statistical analysis was performed using a Student's t test. A p value < 0.05 was considered significant.

Results

Body Weight, Cell Count, and 1,25-(OH)₂D₃ Levels

Pairs of D+ and D- rats were sacrificed at 9 to 12 weeks and studied in parallel. Body weight of D- rats (398.8 ± 74.5 g) was significantly less than that of D+ rats (501.2 ± 79.7 g) ($p < 0.05$, $n=9$ pairs), but they exhibited no mortality or apparent morbidity. There were no differences in the numbers of macrophages obtained by lavage from D+ (AM, $13.7 \pm 9.2 \times 10^6$ per animal; PM, $27.3 \pm 10.7 \times 10^6$) and D- rats (AM, $15.8 \pm 10.5 \times 10^6$; PM, $17.7 \pm 6.7 \times 10^6$) ($p=s$, $n=9$ pairs). As expected,³² 1,25-(OH)₂D₃ levels in serum from D- rats (1.8 pg / ml or 0.5×10^4 pg / μ g of protein) were markedly less than from D+ rats (33.9 pg / ml or 9×10^4 pg / μ g of protein). 1,25-(OH)₂D₃ levels in BAL and peritoneal lavage fluids were measured from pooled samples obtained from a separate group of 4 normal control Sprague-Dawley rats, and in vivo vitamin D₃ concentrations calculated by correcting for estimated volumes of alveolar lining fluid³⁰ and peritoneal lining fluid.³ The calculated 1,25-(OH)₂D₃ concentrations in BAL fluid in normal control rats was 88 pg / ml (2.6-fold that of serum) and peritoneal lavage fluid was 56 pg / ml (1.6-fold that of serum).

Reduced 5-LO Metabolism of AA in AM and PM from Vitamin D₃ Deficient Rats

Having previously shown that in vitro exposure of PBM to the differentiating agent 1,25-(OH)₂D₃ upregulated LT synthetic capacity and FLAP expression,⁵ we were interested in determining whether 1,25-(OH)₂D₃ might have a physiological role in regulating these processes during macrophage differentiation in vivo. This was addressed by studying mature resident AM and PM obtained from D+ and D- rats. Following A23187 stimulation, D+ AM elaborated free AA, 12-(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (12-HETE), and the 5-LO products LTB₄ and 5-(S)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), and small amounts of COX products (PGE₂, thromboxane [Tx] B₂, and HHT [12-hydroxyheptadecatrienoic acid]) were also seen, as expected²⁶ (Figure 1A). Stimulated AM from D- animals released similar amounts of total radioactivity as did those from D+ animals (data not shown). Amounts of 12-HETE and of COX products were also similar in cells from D- animals, but they released 65% less LTB₄ and 67% less 5-HETE than did AM from D+ animals (Figure 1A). An increase in unmetabolized AA in AM from D- animals (82% of total radiolabel) as compared to D+ AM (68%) suggests a reduced ability to metabolize the available endogenous AA. The reduction in 5-LO metabolism of AA in AM from D- animals was confirmed by immunoassay of LTB₄ (Figure 1B) ($n=4$ pairs, $p < 0.05$).

PM were examined in similar fashion. HPLC analysis of A23187-stimulated products elaborated by prelabeled PM from D+ rats indicated

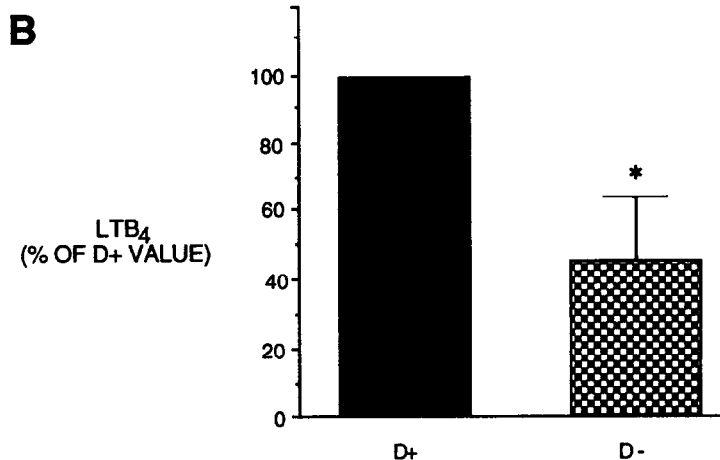
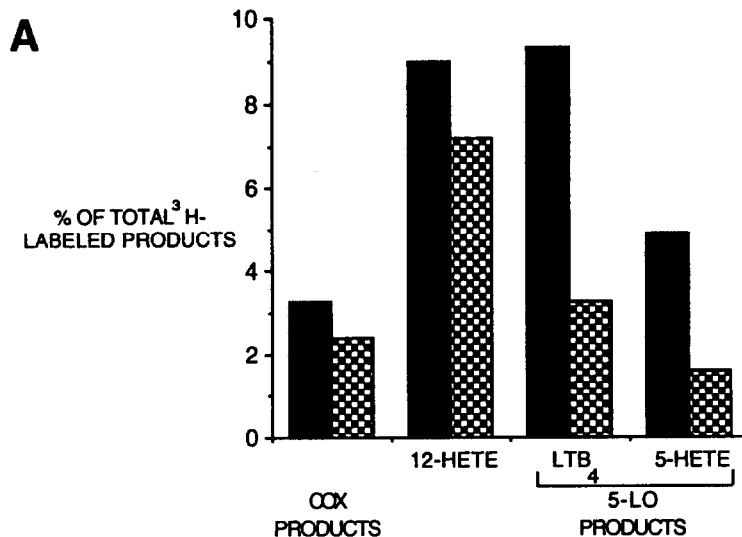


FIGURE 1. Eicosanoid synthesis in AM from D+ and D- rats. (A) AM from D+ and D- rats were adhered for 1 h in M199 and lipids labeled during overnight incubation with [³H]AA. Cells were stimulated with A23187 (1 μM) for 30 min and radiolabeled eicosanoids separated by HPLC analysis, as described in the "Materials and Methods." For each metabolite or group of metabolites, data for D+ (solid bars) and D- (checked bars) are expressed as the percent of total ³H-labeled products eluted. "COX products" include TxB₂, PGE₂, and HHT. "5-LO products" include LTB₄ and 5-HETE. (B) Unlabeled AM from D+ and D- rats were adhered for 1 h in M199, washed and stimulated with A23187. Medium was then harvested for quantitation of immunoreactive LTB₄. Data for cells from D- (checked bars) rats are expressed as a percentage of the paired D+ (solid bars) value (28.13 ± 12.29 ng/ml) and represent the mean ± S.E.M. from 4 pairs of rats; *p < 0.05.

a predominance of COX metabolites, including PGE₂, TxB₂, 6-keto PGF_{1 α} , and HHT, over 5-LO products 5-HETE and LTB₄, as previously reported.²⁶ Small amounts of 12-HETE were also synthesized. Stimulated PM from D- animals released similar amounts of total radioactivity as did those from D+ animals (data not shown). PM from D- animals generated similar quantities of COX and 12-LO products, but 35% less 5-LO products, than did PM from D+ rats (Figure 2A). Likewise, the reduction in 5-LO metabolic capacity of AA in PM from D- animals was confirmed by EIA of LTB₄ (Figure 2B) (n=4 pairs, p <0.05).

Reduced FLAP Expression in AM from Vitamin D₃ deficient Rats

AM from D+ and D- rats were compared for 5-LO and FLAP expression. Figure 3 presents representative autoradiographs, as well as mean densitometric analysis of such data from 4 pairs of rats. Lysates of fresh AM from both groups of rats contained 5-LO protein in both soluble and particulate fractions, as we have reported previously for normal resting rat AM.⁶ There was no difference in the subcellular distribution of 5-LO between D+ and D- AM. Both subcellular fractions were thus analyzed by densitometry and combined to give the mean quantitative data. There was no difference in 5-LO expression in AM from D+ and D- rats (Figure 3A). FLAP, by contrast, was only detected in the particulate fraction. Notably, cells from D- rats exhibited a $52 \pm 14.6\%$ reduction in FLAP protein compared to D+ AM (Fig. 3B) (p < 0.05)

In selected experiments, AM from D- rats were incubated overnight with 50 nM exogenous 1,25-(OH)₂D₃. The addition of exogenous 1,25-(OH)₂D₃ restored LTB₄ synthetic capacity towards the level observed in D+ cells (Figure 4B), and caused a corresponding increase in FLAP expression (Figure 4A). These data strongly suggest that the defects in 5-LO metabolism of AA and FLAP expression in D- AM were indeed related to 1,25-(OH)₂D₃ deficiency.

Likewise, the expression of 5-LO and FLAP was determined in PM from D+ and D- rats. As previously reported,⁶ D+ PM demonstrated a trend towards lower levels of 5-LO protein expression than D+ AM (5-LO expression in PM as a % of AM: $62.2 \pm 44.6\%$, n=3, p=s). FLAP expression was also comparable in the two cell types (FLAP expression in PM as a % of D+ AM: $156.8 \pm 86.2\%$, n=3, p=s), as previously demonstrated.⁵ There was no significant difference in the subcellular distribution of 5-LO in PM from D- rats compared to D+ rats (data not shown). There was no reduction (71% of D+) in 5-LO expression in AM from D- rats (data not shown). Similarly, cells from D- rats exhibited no reduction (97.1 \pm 14.2% of D+) in FLAP protein compared to D+ PM.

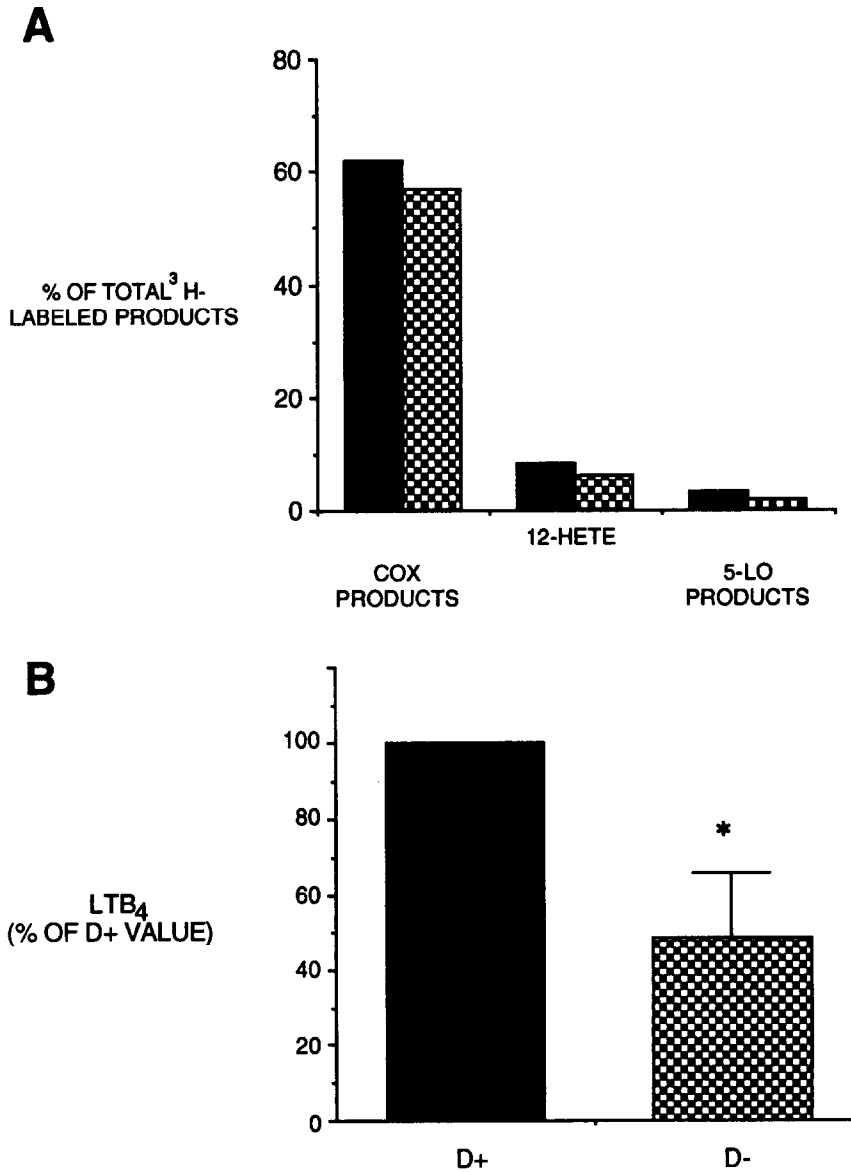


FIGURE 2. Eicosanoid synthesis in PM from D+ and D- rats. (A) PM from D+ and D- rats were adhered for 1 h, labeled overnight with [³H]AA, and stimulated with A23187. Quantitation of individual metabolites or groups of metabolites for D+ (solid bars) and D- (checked bars) are expressed as described in the legend to Figure 1. "COX products" include TxB₂, PGE₂, 6-keto PGF_{1 α} , and HHT. "5-LO products" include LTB₄ and 5-HETE. (B) Unlabeled PM from D+ and D- rats were adhered, stimulated with 1 μ M A23187 for 30 min and the media harvested for quantitation of immunoreactive LTB₄. Data for cells from D- rats are expressed as a percentage of the paired D+ value (3.53 ± 2.7 ng/ml) and represent the mean \pm S.E.M. from 4 pairs of rats; *p < 0.05.

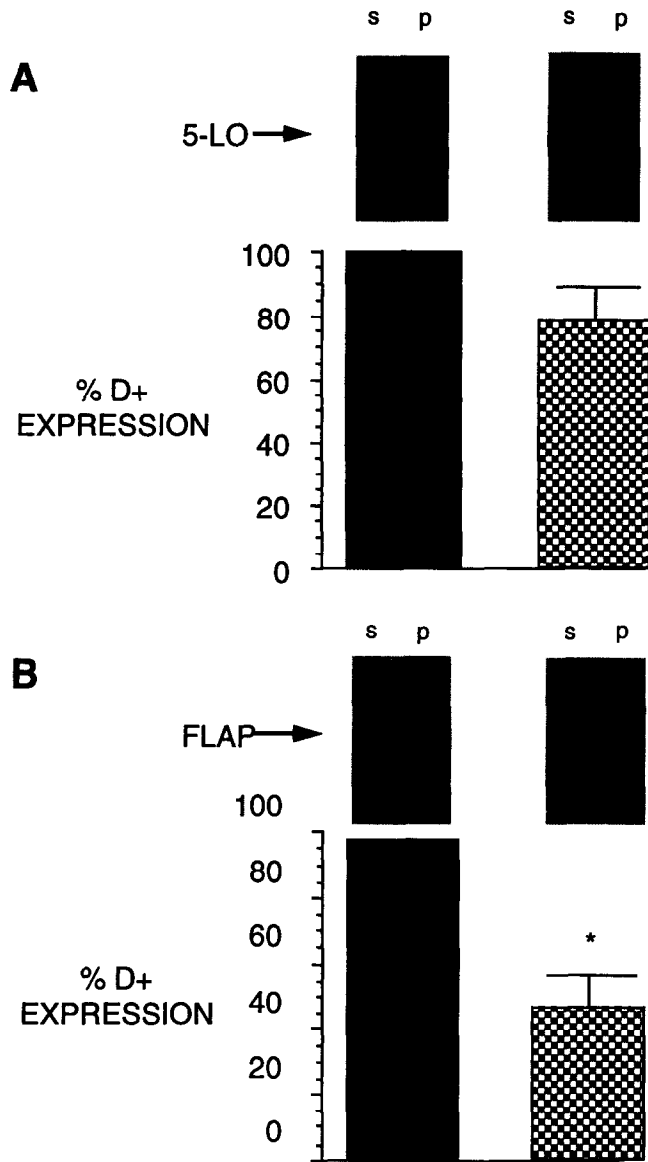


FIGURE 3. 5-LO and FLAP expression in AM from D+ and D- rats. Equal amounts of soluble (s) and particulate (p) proteins (5–20 μ g) obtained from AM of D+ and D- rats were subjected to immunoblot analysis of 5-LO (A) and FLAP (B) as described in the "Materials and Methods." Upper panels show representative autoradiographs from a single pair of rats. Lower panels demonstrate relative levels of 5-LO (soluble plus particulate fractions) (A) and FLAP (particulate fraction only) (B) in cells from both groups of animals as analyzed by video densitometry. Levels in cells from D- rats are expressed as a percentage of the paired D+ value and represent the mean \pm S.E.M. from 4 pairs of rats; * $p < 0.05$.

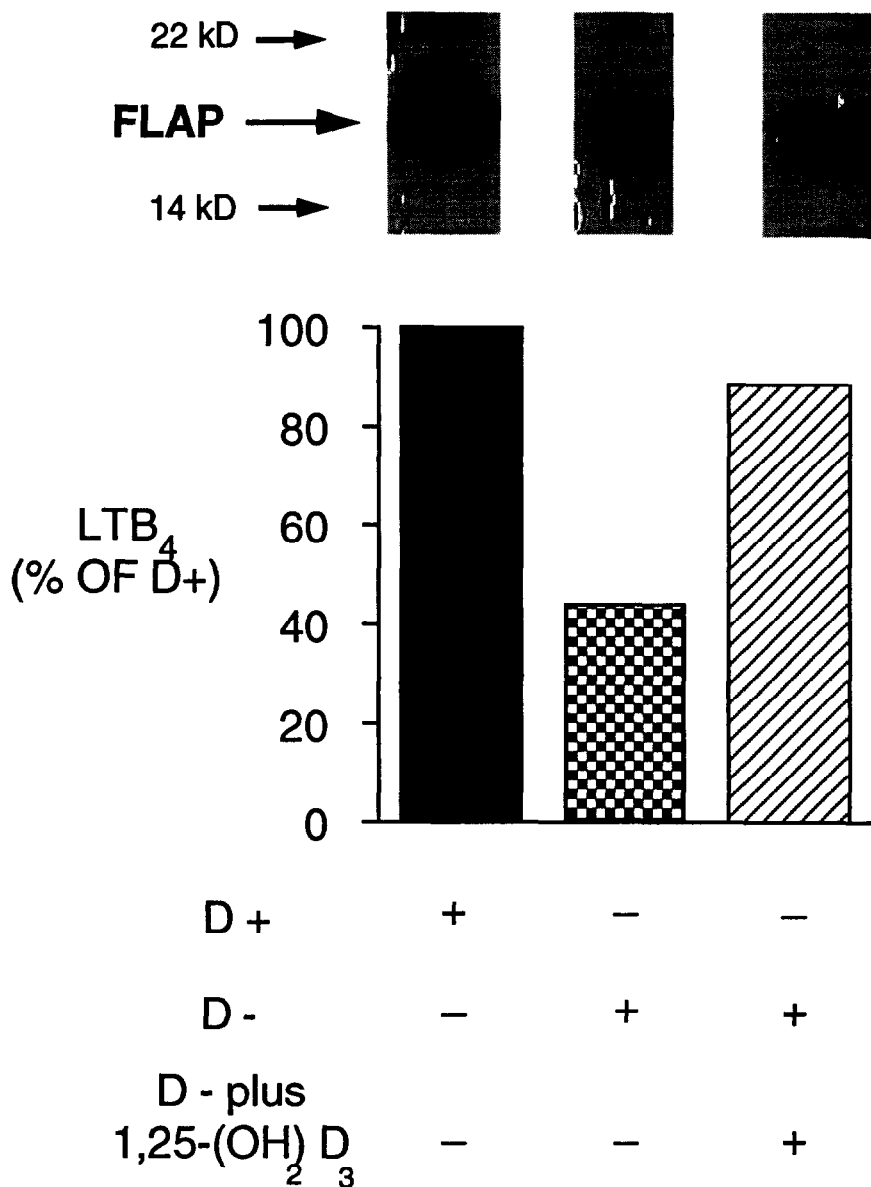


FIGURE 4. Effect of exogenous vitamin D₃ on FLAP expression and LTB₄ synthesis in AM from D- rats. AM from D+ and D- rats were adhered in M199 for 1 h, washed and then cultured overnight in M199 containing 10% FCS with or without 1,25-(OH)₂D₃ (50 nM). (A) Autoradiograph depicting immunoblot analysis of FLAP. (B) A23187-induced (1 μM for 30 min) LTB₄ synthetic capacity under the same conditions. In this experiment AM from D+ rats (3.9 ng/ml) synthesized greater amounts of LTB₄ than AM from D- rats (1.7 ng/ml), and this was almost completely restored (3.5 ng/ml) by overnight incubation with 1,25-(OH)₂D₃.

COX Protein Expression in Macrophages and Type II AEC is Similar in D+ and D- Rats

As indicated above, there was no significant difference in COX metabolism of AA in AM or PM from D- as compared to D+ rats. To extend this finding, expression of COX proteins was determined in AM and PM from D+ and D- rats. COX-2 was not detected in fresh AM or PM, which is consistent with its inducible role,¹¹ but COX-1 was expressed in resting cells. Confirming the metabolic data, there was no reduction in COX-1 protein expression in either macrophage cell type obtained from D- rats (AM, $114.7 \pm 27.6\%$ of D+; PM, $92.8 \pm 18.5\%$ of D+, n=5). Figure 5A demonstrates a representative immunoblot analysis of COX-1 and COX-2 proteins in AM from D+ and D- rats. Type II AEC line the alveolar surface, and have a high capacity for prostaglandin synthesis.¹⁹ Therefore, expression of COX proteins was examined in type II AEC isolated from D+ and D- rats and harvested after 48 h in culture. These cells expressed both COX-1 and COX-2 proteins. There was no reduction in COX-1 or -2 expression in cells from D- animals (126% and 118% of D+, respectively, n=2) (Figure 5B). 5-LO and FLAP expression were not detected (data not shown).

Discussion

We have previously demonstrated that PBM exhibit reduced FLAP expression compared to differentiated macrophages,⁷ and that $1,25\text{-}(\text{OH})_2\text{D}_3$ -differentiated PBM exhibit a parallel increase in 5-LO metabolism of AA and FLAP expression.⁵ An increase in LT synthetic capacity in HL-60 cells incubated with $1,25\text{-}(\text{OH})_2\text{D}_3$ has also been reported.⁴ Consequently, the present study was performed to evaluate the physiological role of $1,25\text{-}(\text{OH})_2\text{D}_3$ in regulating eicosanoid metabolism in mononuclear phagocytes. For comparison, type II AEC were studied. We examined both 5-LO and COX pathways in addition to the expression of 5-LO, FLAP, COX-1, and COX-2 proteins. Several important findings emerged from this study. 1) AM from D- rats demonstrated parallel reduction in 5-LO metabolism of AA and FLAP (but not of 5-LO) expression compared to AM from D+ rats. 2) 5-LO metabolism of AA was also reduced in PM from D- rats, albeit without any significant change in FLAP expression. 3) The physiological role of vitamin D₃ appears to be specific for the 5-LO pathway, since its deficiency did not affect overall AA release in macrophages, the COX metabolic pathway or COX protein expression in either macrophages or type II AEC, or the 12-LO pathway in macrophages. 4) $1,25\text{-}(\text{OH})_2\text{D}_3$ concentrations in BAL and peritoneal lavage fluids exceeded that in serum, which may help explain the relatively greater FLAP expression in mature AM and PM than in precursor PBM.

A unique feature of this study was our ability to address a possible in

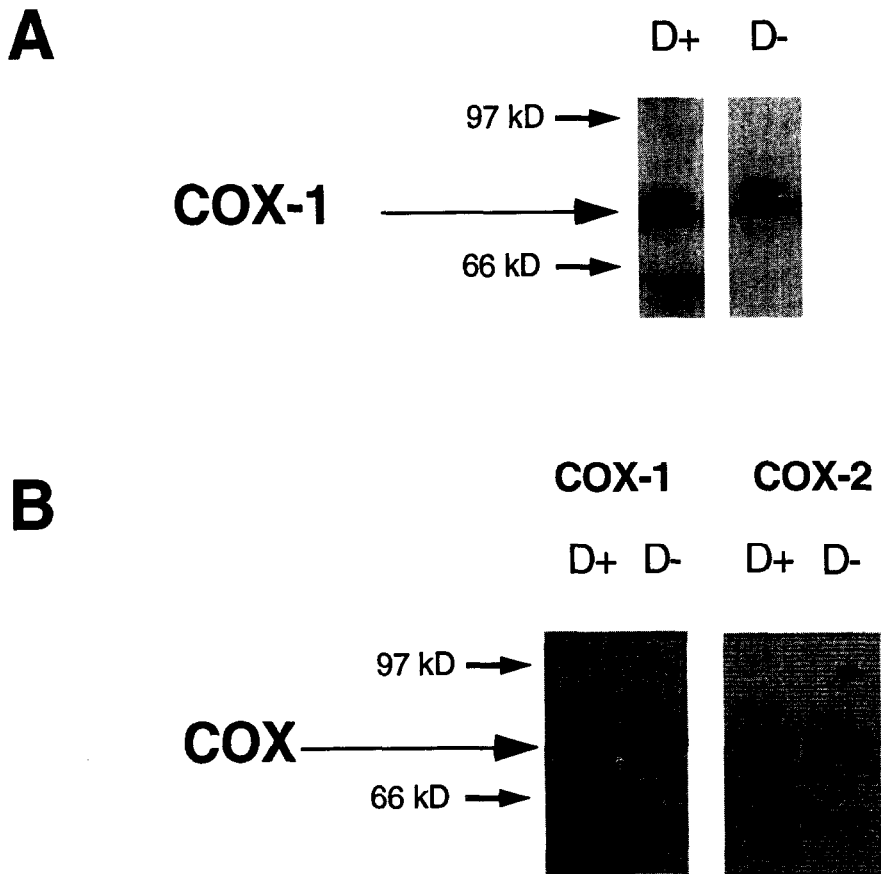


FIGURE 5. COX protein expression in AM and type II AEC from D+ and D- rats. (A) Particulate proteins (7 μ g) obtained from AM from D+ and D- rats were subjected to immunoblot analysis for COX-1, as described in the "Materials and Methods." (B) Particulate fractions (20 μ g) obtained from type II AEC from D+ and D- rats were subjected to immunoblot analysis for both COX-1 and COX-2, as described in the "Materials and Methods."

vivo role for 1,25-(OH)₂D₃ in regulating eicosanoid metabolism in macrophages by making use of an established rat model of vitamin D₃ deficiency. Determination of 1,25-(OH)₂D₃ levels in the serum verified the deficiency state of the experimental model. Interestingly, 1,25-(OH)₂D₃ levels in normal rats were 2.6-fold higher in BAL fluid and 1.6-fold higher in peritoneal lavage fluid than in serum. Greater 1,25-(OH)₂D₃ levels in these compartments than in serum might account for the greatly increased FLAP expression in AM and PM compared to PBM.⁵ The mechanism underlying the greater 1,25-(OH)₂D₃ levels in BAL and peritoneal lavage

fluid than in serum is unknown. Notably, resident AM from D- animals demonstrated a selective decrease in LT synthetic capacity which was associated with a significant decrease in FLAP expression, as compared to cells from matched D+ rats. The magnitude of reduction in 5-LO metabolism of AA and in FLAP expression were quantitatively similar (i.e., ~50% of D+ levels). That these defects were indeed a consequence of 1,25-(OH)₂D₃ deficiency was confirmed by showing that they were largely corrected by the addition of exogenous 1,25-(OH)₂D₃.

As compared to cells from normal rats, PM from D- rats exhibited a reduction in 5-LO metabolism of AA which was of similar magnitude to that observed in AM. However, this was not associated with a significant reduction in FLAP expression. These data in PM do not conform neatly to the hypothesis that FLAP expression is a function of 1,25-(OH)₂D₃ levels. An alternative explanation for this finding in PM includes the possible effect of vitamin D₃ deficiency on other factors, apart from FLAP or 5-LO expression, which might regulate the 5-LO pathway. 1,25-(OH)₂D₃ is known to augment intracellular Ca²⁺ levels²³ and the transcription²⁴ as well as activation of protein kinase C.^{29,31} Reduction in any of these effects might be expected to decrease the level of 5-LO metabolism of AA within the cell since LT synthetic capacity is dependent on intracellular Ca²⁺ concentrations²⁸ and levels of active protein kinase C.²⁵

The effect of 1,25-(OH)₂D₃ appears to be specific for the 5-LO pathway, as vitamin D₃ deficiency did not affect the total quantity of AA release or the metabolism of AA via the 12-LO or COX pathways in macrophages. The lack of effect on the COX pathway was confirmed by immunoblot analysis for COX-1, the constitutively expressed COX isoform, in AM and PM. COX-2 was not detected in fresh AM or PM. In type II AEC, which express COX-1 and COX-2, neither COX isoform was reduced in D- as compared to D+ animals. This is in keeping with our previous data with 1,25-(OH)₂D₃-differentiated PBM, in which there was no significant effect on PG synthesis.⁵ By contrast, it has been previously reported that 1,25-(OH)₂D₃ increased COX metabolism of AA in HL-60 cells.¹⁴ These results, which differed from those presented here, might be explained by differences in the cell type studied (malignant HL-60 cell line) or in culture conditions (lower 1,25-(OH)₂D₃ concentration, and serum-free Isocove's modified Dulbecco's medium).

In summary, our data strongly indicate that 1,25-(OH)₂D₃ is not merely capable of upregulating 5-LO metabolism of AA by increasing FLAP expression, as previously reported,⁵ but that 1,25-(OH)₂D₃ plays a *physiological role* in maintaining FLAP expression and the 5-LO pathway in mature AM in vivo. Considering the role of 5-LO products, especially LTB₄, as chemotactic factors important in normal host defense,²⁰ this finding provides a possible explanation for the increased susceptibility to infection which has been recognized in association with 1,25-(OH)₂D₃ deficiency.^{2,12}

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Abbreviations

AA, arachidonic acid; AEC, alveolar epithelial cell; AM, alveolar macrophage; BAL, bronchoalveolar lavage; BSA, bovine serum albumin; COX, cyclooxygenase; DMEM, Dulbecco's modified Eagles' medium; DMSO, dimethyl sulfoxide; EIA, enzyme immunoassay; FLAP, 5-lipoxygenase activating protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 5-HETE, 5-(S)-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-(S)-hydroxy-6,8,11,14-eicosatetraenoic acid; HHT, 12-hydroxyheptadecatrienoic acid; HL-60 cells, human lymphoma 60 cells; HPLC, high performance liquid chromatography; 5-LO, 5-lipoxygenase; 12-LO, 12-lipoxygenase; LTB₄, leukotriene B₄ [(5S,12R)-dihydroxy-6,14-cis-8,14-trans-eicosatetraenoic acid]; PBM, peripheral blood monocyte; PBS, phosphate buffered saline; PGE₂, prostaglandin E₂; PM, peritoneal macrophage; SDS, sodium dodecyl sulfate; TxB₂, thromboxane B₂; TBS, Tris buffered saline; 1,25-(OH)₂D₃, 1,25-dihydroxy vitamin D₃; normal/control rats (D+); vitamin D₃-deficient (D-).

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