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Molecular mechanisms involved in the enhancement of mitochondrial malate dehydrogenase activity by calcitriol in chick intestine☆

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Abstract

Mitochondrial malate dehydrogenase (mMDH) from the intestine is the NAD-linked oxidoreductase of the tricarboxylic acid cycle with the highest activity and response to vitamin D treatment in vitamin D-deficient chicks (−D). The aim of this study was to elucidate potential molecular mechanisms by which cholecalciferol or calcitriol enhances the activity of this enzyme. One group of animals used was composed of −D and −D treated with cholecalciferol or with calcitriol. A second group consisted of −D and −D supplemented with high Ca^{2+} diet. A third group included chicks receiving either a normal or a low Ca^{2+} diet. In some experiments, animals were injected with cycloheximide. Data showed that either vitamin D (cholecalciferol or calcitriol) or a low Ca^{2+} diet increases mMDH activity. High Ca^{2+} diet did not modify the intestinal mMDH activity from −D. The mMDH activity from −D remained unaltered when duodenal cells were exposed to 10^{-8} mol/L calcitriol for 15 min. The enhancement of mMDH activity by calcitriol was completely abolished by simultaneous cycloheximide injection to −D. mMDH mRNA levels, detected by RT-PCR, indicate that calcitriol did not affect gene expression. In contrast, Western blots show that calcitriol enhanced the protein expression. In conclusion, calcitriol stimulates intestinal mMDH activity by increasing protein synthesis. No response of mMDH activity by rapid effects of calcitriol or activation through increment of serum Ca^{2+} was demonstrated. Consequently, ATP production would be increased, facilitating the Ca^{2+} exit from the enterocytes *via* the Ca^{2+}-ATPase and Na^{+}/Ca^{2+} exchanger, which participate in the intestinal Ca^{2+} absorption.

Keywords: Malate dehydrogenase; Calcitriol; Intestine; Mitochondria; Vitamin D-deficient chicks; Low Ca diet

1. Introduction

Cholecalciferol or vitamin D_3 is hydroxylated in two steps: the first hydroxylation occurs in the C-25 in the liver, and the second hydroxylation takes place in kidney at the level of C-1. Calcitriol or 1,25-dihydroxyvitamin D_3, the resulting product of these two hydroxylations, is the major regulatory hormone of intestinal calcium absorption, a process that occurs through paracellular and transcellular pathways. The latter comprises three steps of Ca^{2+} transport: entry across the brush border membrane, intracellular diffusion and exit through the basolateral membrane. All genes presumably involved in the transcellular pathway are enhanced by calcitriol [1]. Low Ca^{2+} diets produce the same effect, probably by activation of the vitamin D endocrine system [2,3]. The mechanism by which calcitriol enhances the transcellular pathway involves activation of a vitamin D receptor (VDR), a member of a nuclear receptor superfamily. Paracellular Ca^{2+} transport across intestinal epithelial cells has been suggested to be also promoted by vitamin D_3 [4]. Recently, Fujita et al. [5] have shown that tight junction proteins claudin-2 and -12 are up-regulated in enterocytes by calcitriol through VDR activation. The authors suggest that both proteins form paracellular Ca^{2+} channels in intestinal epithelia, providing a novel mechanism underlying vitamin D-dependent intestinal Ca^{2+} absorption. Tudpor et al. [6] have demonstrated that calcitriol uses the nongenomic signaling pathways involving PI3K, PKC and MEK to rapidly enhance the solvent drag-induced calcium transport in the duodenum of female rats.

The movement of the cation through the transcellular pathway occurs with energy expenditure. ATP hydrolysis is involved in the exit of Ca^{2+} ions from enterocytes to the lamina propria, which is performed either by the plasma membrane Ca^{2+}-ATPase, also named Ca^{2+} pump (PMCA), or Na^{+}/Ca^{2+} exchanger (NCX1) [7]. The tricarboxylic acid (TCA) cycle is the major final common pathway for oxidation of different substrates, which produce reducing equivalents in the form of nicotinamide adenine dinucleotide and flavin adenine dinucleotide that result in production of large amounts

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of ATP *via* oxidative phosphorylation. The activity of all NAD-linked oxidoreductases from the TCA cycle in the intestinal mucosa was found to be increased by vitamin D treatment to vitamin D-deficient chicks. Mitochondrial malate dehydrogenase (mMDH) (EC 1.1.1.37) is the NAD-linked oxidoreductase with the highest activity and response to vitamin D treatment in vitamin D-deficient chicks [8]. It catalyzes the dehydrogenation of L-malate to oxaloacetate in the final step of TCA cycle. mMDH expression is encoded by a nuclear gene and is not a "constitutive" protein, but rather exhibits developmental, stage-specific and tissue-specific regulation [9]. However, the mechanism by which calcitriol regulates the mMDH activity has not been reported in the literature.

Therefore, the aim of this study was to elucidate potential molecular mechanisms by which calcitriol enhances intestinal mMDH activity from vitamin D-deficient chicks, which could contribute not only to the understanding of intestinal Ca^{2+} absorption stimulated by vitamin D, but also to a better knowledge of other biological responses provoked by calcitriol in the chick intestine.

2. Material and methods

One-day-old Cobb Harding chicks (*Gallus domesticus*) were obtained from Gavipor SA (Río Ceballos, Córdoba, Argentina) and were fed a rachitogenic diet [10], modified to contain 1% calcium and 0.8% phosphorus concentrations. At 4–5 weeks of age, the animals were vitamin D deficient (−D), as judged by impaired growth and low serum Ca^{2+} concentrations. The animals were divided into two groups: (a) −D chicks (control group) and (b) −D chicks treated orally with 2000 IU of cholecalciferol (Sigma-Aldrich, St. Louis, MO, USA) in propylene glycol or intraperitoneally with 1 μg of calcitriol (donated generously by Leo Pharma, Ballerup, Denmark), 24 and 12 h before experiment, respectively. A second group of 1-day-old chicks were divided into another two groups and each group was fed for 4–5 weeks one of the following diets: (a) rachitogenic diet (control group) and (b) rachitogenic diet supplemented with 4% $CaCO_3$ (high Ca^{2+} diet). A third group of 1-day-old chicks received a starter commercial normal avian diet (Cargill, SA, Pilar, Córdoba, Argentina) for 3 weeks. At this time, the chicks were divided into two groups and each group was fed for 10 additional days one of the following diets: (a) control diet (1% Ca, 0.8% P) and (b) low Ca^{2+} diet (0.1% Ca, 0.8% P), as previously described [11], and both diets contained 1200 IU of cholecalciferol per kilogram. In some experiments, vitamin D-deficient chicks were injected with cycloheximide (100 μg/kg of body weight) or with cycloheximide and calcitriol at the same time. The protocols were conducted according to the Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering. Blood samples were taken by cardiac puncture for determination of serum calcium by atomic spectrometry. Then, the animals were killed by cervical dislocation, and the excised duodenae were rinsed with cold 0.15 mmol/L NaCl, and cells or mitochondria were isolated as described below.

2.1. Duodenal cell isolation

Intestinal epithelial cells were isolated from chick duodenum by enzymatic procedure. Each duodenum was immersed in 0.15 mmol/L NaCl at 4°C and closed by one extreme forming a duodenal sac. One milliliter of isolation buffer composed of 118 mmol/L NaCl, 5.3 mmol/L KH_2PO_4, 8.3 mmol/L Na_2HPO_4, 1.5 mmol/L KCl (pH 7.4) and 1 mg hyaluronidase was introduced into the sac, which was closed by the other end and incubated at 37°C for 30 min under constant moderate shaking. At this time, the duodenum sac was removed from the bath, cut and the cells were pelleted by centrifugation at 200×*g* for 5 min. After rinsing twice in the same medium free of hyaluronidase, cells were resuspended in the same solution until obtaining a concentration of 5–6 g of protein per liter. Cellular viability was assayed by the Trypan blue exclusion technique.

2.2. Isolation of intestinal mitochondria

Mitochondria were isolated from intestinal mucosa or duodenal cells of each group of animals by differential centrifugation as previously described [12]. Protein was determined by the method of Gornall et al. [13]. Enzymatic characterization of intestinal mitochondria was as reported by Tolosa de Talamoni et al. [12].

2.3. Mitochondrial malate dehydrogenase assay

mMDH (EC 1.1.1.37) was assayed in supernates of mitochondrial extracts (5 mg of protein) prepared by freezing and thawing the mitochondria three times in 500 μl of 100 mmol/L potassium phosphate buffer (pH 7.2) containing 5 mmol/L 2-mercaptoethanol and centrifuging for 10 min at 10,000×*g* in an Eppendorf 3200 microfuge. The pellet contained less than 1% of the activity of the supernates. The assay medium contained 0.1 mmol/L phosphate buffer (pH 7.4), 3.3 mmol/L NADH and 0.1 mmol/L oxalacetate. The oxidation of NADH was followed at 37°C at 340 nm (E=6.23×10^3). The results are expressed in micromoles of NAD formed per milligram of protein per minute.

2.4. Western blot analysis

mMDH expression was analyzed by Western blot procedure. Briefly, proteins (50 μg) from homogenates of intestinal mitochondria were separated by SDS-PAGE with 12% acrylamide and 0.1% SDS [14] and transferred to nitrocellulose sheets [15]. Equal protein loading in each lane was confirmed by staining of mitochondria with a Ponceau reagent [16]. The blots were incubated with a polyclonal rabbit antiserum against purified *Trypanosoma cruzi* mMDH (1:1000 dilution) (donated generously by Dr. Nowicki, University of Buenos Aires, Argentina), at 4°C overnight. The secondary antibody was a peroxidase-conjugated goat antibody against a broad spectrum of antigens (Histostain bulk kit, Zymed Lab, San Francisco, CA, USA), which was incubated at room temperature for 1.5 h. Nonspecific binding on the nitrocellulose was blocked with nonfat dry milk in Tris-buffered saline solution. Detection was performed by using diaminobenzidine solution as chromogen. The band intensities were quantified using the KSLite version 2.0 software (Kontron Elektronik GmbH, Eching, Germany) in order to obtain the relative expression of proteins.

2.5. RNA isolation and RT-PCR analysis

Total RNA isolation was performed with TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined by spectrophotometry. cDNA was synthesized by using 400 U of M-MLV reverse transcriptase (Invitrogen) in buffer [50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 20 mmol/L dithiotreitol], containing each deoxynucleotide triphosphate at 2 mmol/L, 40 U RNase inhibitor (RNaseOUT, Invitrogen) and 500 ng random primers. mMDH PCR amplification was done on cDNA using 1.25 U Taq polymerase in a buffer containing 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.4) and 2 mmol/L $MgCl_2$, with 0.8 mmol/L of each deoxynucleotide triphosphate and 0.8 μmol/L each of two oligonucleotide primers. Conditions for mMDH PCR amplification were as follows: denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 1 min, for a total of 35 cycles, with an extension step of 10 min at 72°C in the final cycle. Primer sequences for mMDH PCR amplification were as follows: forward (5′-CAGCTGCCAGAATGTTTGAA-3′) and reverse (5′-ATGGCCACCAATAACTGG AA-3′), which amplify a 348-bp region localized between 292 and 639 bp. Human 18S ribosomal RNA was used as internal control. Primer sequence was follows: forward (5′-GTAACCCGTTGAACCCCATT-3′) and reverse (5′-CCATCCAATCGGTAGTAGC.

G-3′); the PCR product obtained was 131 bp long. PCR products were run on a 1.5% agarose gel prestained with ethidium bromide and visualized under UV light. mMDH mRNA levels were normalized to the expression of 18S ribosomal RNA level in each sample.

2.6. Ca^{2+} uptake by enterocytes

The procedure of Ca^{2+} uptake by enterocytes described by Liang et al. [17] was used with slight variations. An aliquot of 300 μL of cell suspension (about 5 g of protein/L) was incubated with an uptake solution composed of 140 mmol/L KCl, 10 mmol/L HEPES, 2 mmol/L $CaCl_2$ (pH 7.4) plus 1.85×10^8 Bq/L of $^{45}CaCl_2$. At 15 min, the Ca^{2+} uptake was stopped with 1 ml of the same solution free of $^{45}Ca^{2+}$ plus 2 mmol/L EGTA. The mixture was centrifuged for 1 min at 10,000×*g*, which was repeated. The final pellet was resuspended in 1 mmol/L NaOH. Radioactivity was measured in a liquid scintillation counter. Results are expressed as nanomoles of Ca^{2+} per milligram of protein.

2.7. Chemicals

$^{45}Ca^{2+}$ was purchased from NEN (Boston, MA, USA). NADH, oxalacetate and other chemicals were of reagent grade and obtained from Sigma-Aldrich.

2.8. Statistical analysis

Data are expressed as means±standard errors. Results were evaluated by one-way analysis of variance, and Tukey test was used as a *post hoc* analysis. Student's *t* test was also used to compare means. Differences were considered significant at P<.05.

3. Results

As expected, either oral administration of 2000 IU of vitamin D or intraperitoneal injection of 1 μg of calcitriol to 4-week-old −D chicks increased the intestinal mMDH activity. This effect was observed after 24 h of vitamin D administration or 12 h after calcitriol injection (Fig. 1A). Low Ca^{2+} restriction for 10 days also increased the intestinal

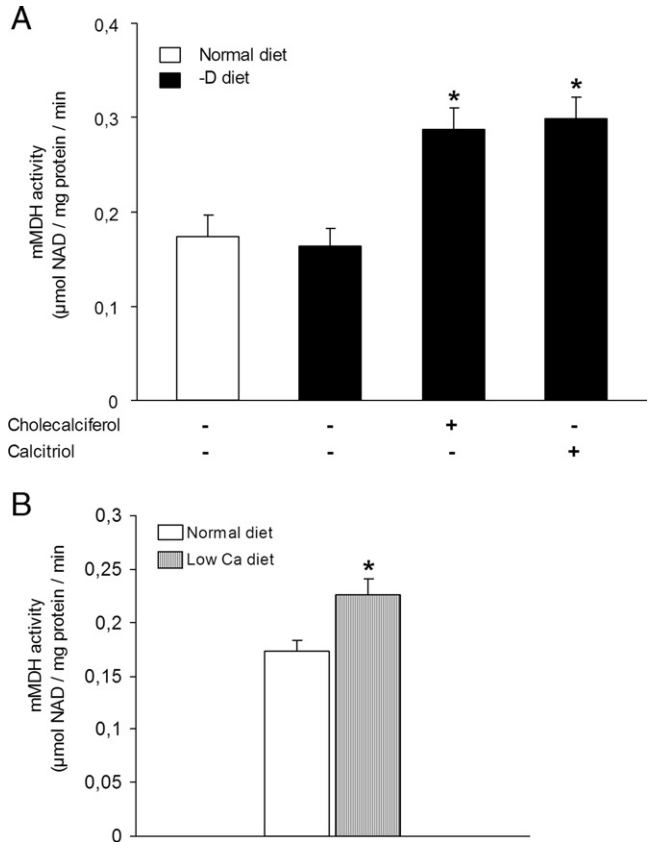

Fig. 1. (A) Effect of oral administration of 2000 IU of vitamin D or intraperitoneal injection of 1 μg of calcitriol on intestinal mMDH activity from −D chicks. (B) Effect of a low calcium diet on intestinal mMDH activity from normal chicks. mMDH activity was assayed as described in Materials and Methods. Results are means±S.E. of four determinations in pools from mitochondrial extracts of three animals each. (A) *P<.05 *vs.* normal and −D chicks. (B) *P<.05 *vs.* normal chicks.

mMDH activity as compared to that from chicks fed a normal avian diet (Fig. 1B).

In order to elucidate whether this effect was due to the secosteroid hormone or to high levels of serum Ca^{2+}, the enzyme activity was analyzed in intestinal mitochondria from −D chicks supplemented with 4% Ca^{2+} in comparison with −D chicks (controls) and with −D chicks treated with calcitriol. High Ca^{2+} diet did not modify the intestinal mMDH activity from −D chicks, although serum Ca^{2+} levels of animals supplemented with high Ca^{2+} were as high as those from −D chicks treated with calcitriol (Fig. 2A and B).

The mMDH activity from −D chicks remained unaltered when duodenal cells were exposed to 10^{-8} mol/L calcitriol for 15 min (Fig. 3A). However, Ca^{2+} uptake by the enterocytes was significantly increased after that hormone exposure (Fig. 3B).

The enhancement of intestinal mMDH activity by calcitriol was completely abolished by simultaneous cycloheximide injection to −D chicks. The enzyme activity was also diminished by cycloheximide injection to −D chicks (Fig. 4).

Semiquantitative analysis of mMDH mRNA levels, performed by RT-PCR procedure, reveals that the mMDH gene expression in chick enterocytes has not been changed by calcitriol treatment. Furthermore, the expression was similar among normal, −D chicks and −D chicks treated with calcitriol (Fig. 5A). In contrast, the protein expression analyzed by Western blot shows that calcitriol enhanced the protein expression after 12 h of administration to −D chicks (Fig. 5B). No statistical differences were observed in the mMDH protein expression between normal chicks and −D chicks.

4. Discussion

This study confirms that either cholecalciferol or calcitriol, hormone derived from vitamin D, stimulates the intestinal mMDH activity from −D chicks, as previously reported [8]. It also shows that the same effect is produced when chicks are fed a low Ca^{2+} diet as compared to those receiving a normal diet, which suggests that the enhancement of intestinal mMDH activity is produced either by exogenous or endogenous calcitriol. When dietary Ca^{2+} is low, serum levels of $1,25(OH)_2D_3$ are enhanced by stimulation of renal 1α-hydroxylase activity in chicks [1,11]. Therefore, the effect of the low Ca^{2+} diet is due, at least partially, to the stimulation of the vitamin D endocrine system [18]. The involvement of vitamin D in energy metabolism is not quite clear [19]. Billaudel et al. [20] demonstrated that vitamin D deficiency severely affected glucose oxidation through the TCA cycle, as measured by $^{14}CO_2$ production from D-[6-^{14}C] glucose in rat endocrine pancreas. The authors did not find a major influence on the first steps in glycolysis. They support that vitamin D deficiency altered the glycolytic pathway after the D-glyceraldehyde step and mainly damaged the oxidative events within the TCA cycle. Stio et al. [21] demonstrated that vitamin D deficiency remarkably inhibited NAD-dependent isocitrate dehydrogenase activity in rat heart, which was restored to the normal value by calcitriol administration. In contrast, vitamin D deficiency produced an

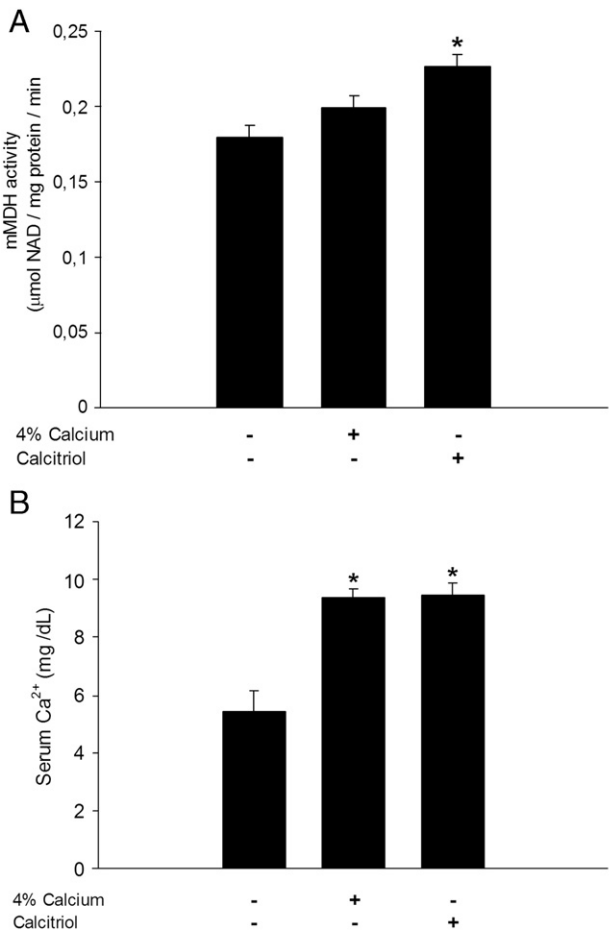

Fig. 2. (A) Influence of high Ca^{2+} diet (4% Ca^{2+}) or intraperitoneal injection of 1 μg of calcitriol on intestinal mMDH activity from −D chicks. Results are means±S.E. of four determinations in pools from mitochondrial extracts of three animals each. (B) Effect of high Ca^{2+} diet (4% Ca^{2+}) or intraperitoneal injection of 1 μg of calcitriol on serum Ca^{2+} of −D chicks. Results are means±S.E. from five animals each group. (A) and (B) *P<.05 *vs.* −D.

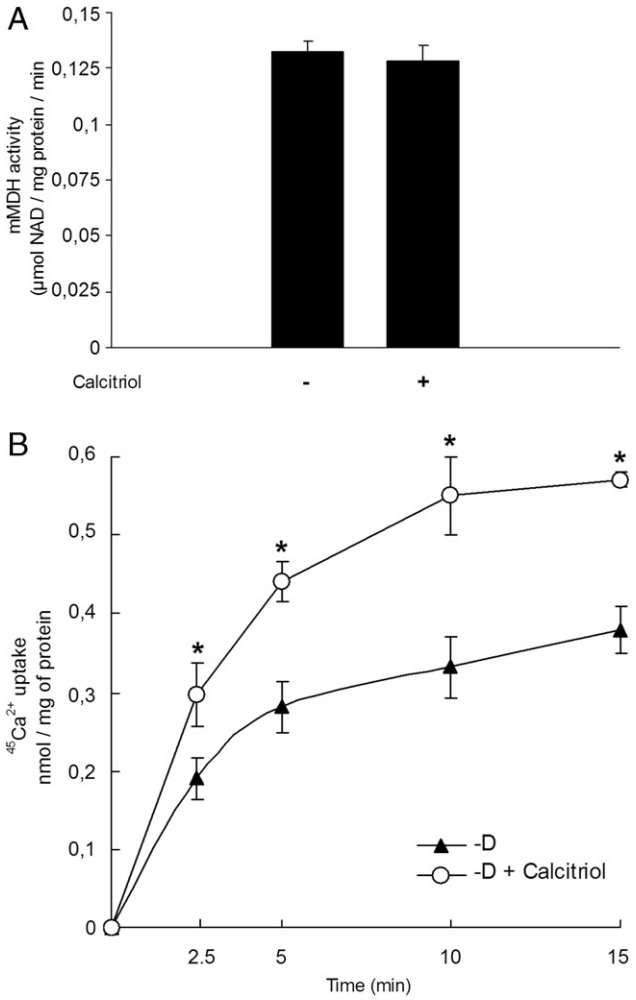

Fig. 3. (A) Intestinal mMDH activity from −D chick enterocytes exposed to 10^{-8} mol calcitriol/L for 15 min. Results are means±S.E. of five determinations in pools of mitochondrial extracts from three animals each. (B) A time course of $^{45}Ca^{2+}$ uptake by enterocytes from −D chicks. Results are means of five determinations from pools of enterocytes from four animals each. *P<.01 *vs.* −D.

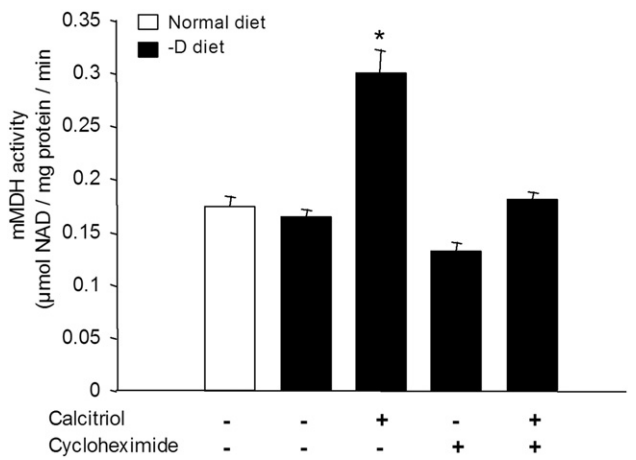

Fig. 4. Effect of intraperitoneal injection of 1 µg of calcitriol and simultaneous 100 µg of cycloheximide injection on intestinal mMDH activity from −D chicks. Results are means±S.E. of five determinations in pools of mitochondrial extracts from three animals each. *P<.05 *vs.* normal and −D.

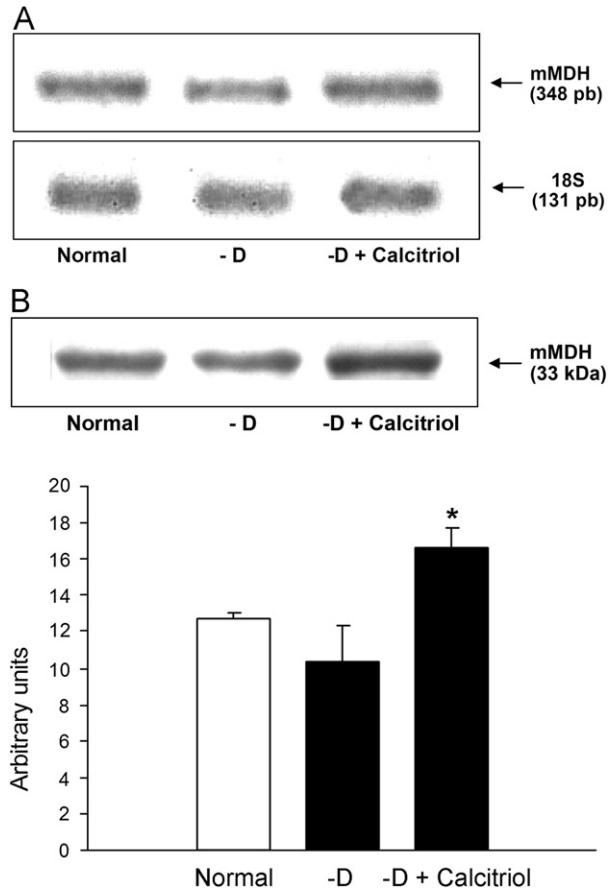

Fig. 5. Effect of intraperitoneal injection of 1 µg of calcitriol on (A) mMDH gene expression in chick enterocytes. Three micrograms of total RNA from duodenal mucosa was subjected to RT-PCR analysis; human 18S ribosomal RNA was used as internal control. (B) Intestinal mMDH protein expression from −D chicks. Western blot analysis represents one of three independent experiments. The molecular weights were calculated by comparison with molecular weight markers run simultaneously with the protein samples. Statistical analysis of the band intensities is represented in the bottom graphic of (B). *P<.05 *vs.* −D.

increase in NADP-isocitrate dehydrogenase activity from rat cerebral cortex, an effect that was not corrected by calcitriol supply [22].

Since the stimulatory effect on mMDH activity could be produced by high serum Ca^{2+} levels triggered by vitamin D_3, the enzyme was assayed in supernatants from intestinal mitochondria of Ca^{2+}-repleted −D chicks. These animals, which had normalized serum Ca^{2+} levels, did not exhibit higher intestinal mMDH activity in comparison with that from −D chicks. In other words, the lack of change in the intestinal mMDH activity by high serum Ca^{2+} is an indication that the vitamin D effect on the enzyme is not through the increment of extracellular Ca^{2+} levels. We previously demonstrated that $k_{0.5}$ values for substrate were not modified by addition of Ca^{2+} ions into the incubation medium [8]. Therefore, neither the affinity of mMDH for its substrate nor the activity of the enzyme is altered by Ca^{2+} ions [23,24].

A rapid nongenomic effect of calcitriol on intestinal mMDH activity seems not to be involved because the short exposure of duodenal cells to physiological hormone concentration did not modify the enzyme activity. However, the same calcitriol concentration increased Ca^{2+} uptake by mature enterocytes, as also shown by other authors [3,25]. By contrast, a slower mechanism appears to be responsible for the enhancement of mMDH activity produced by calcitriol. The abolition of this increase provoked by simultaneous injection of cycloheximide indicates that protein synthesis is involved

in the mechanism. This is also confirmed by the higher mMDH protein expression, detected by Western blots, in samples from −D chicks treated with calcitriol as compared to those from −D chicks. The regulation of mMDH activity by calcitriol would be at the translation step or posttranscriptional and translation step. It has been shown that $1,25(OH)_2D_3$ treatment retards the degradation of IκBα-mRNA in P388D1 cells [26] and increases the stability of CaR mRNA from the kidney of genetic hypercalciuric stone-forming rats [27]. Recently, the calcitriol treatment significantly enhanced MRP4 protein expression by increasing protein stability without affecting mRNA expression in Caco-2 cells, which was confirmed by cycloheximide experiments [28]. The lack of change in the intestinal mMDH mRNA levels suggests that gene transcription is not regulated by the hormone. It is quite possible that the steady-state levels of the transcript are maintained, independently of alterations in the rates of synthesis and degradation.

Another possible mechanism to explain the increment in the mMDH activity by calcitriol would be related to the antioxidant capacity of vitamin D [29]. Oral administration of vitamin D to Sprague-Dawley rats produced an increase in liver glutathione content and in glucose-6-P dehydrogenase, glutathione peroxidase and superoxide dismutase activities as well as a decrease in lipid peroxidation and glutathione reductase activity [30]. All these effects maintain the system in low oxidative stress. Hodges et al. [31] demonstrated that the modifications of essential sulfhydryl residues of mMDH alter the proper binding of NADH. In our laboratory, it has been shown that intestinal mMDH activity is decreased by artificial glutathione depletion caused by menadione administration [32]. Therefore, all the data indicate that mMDH is sensitive to drugs affecting the redox state of the cell. Shi et al. [33] demonstrated in cultures of human adipocytes that calcitriol inhibits the uncoupling protein UCP2 expression *via* nVDR. UCP2 stimulates mitochondrial proton leak, exhibiting a potential role in thermogenesis; hence, UCP2 suppression by calcitriol may contribute to improve the energy efficiency. Recent experiments have shown that VDR-null mice show markedly higher energy expenditure, oxygen consumption and CO_2 production as well as higher expression of uncoupling proteins UCP1, UCP2 and UCP3 in comparison with wild-type mice. The expression of the UCPs has been shown to be directly suppressed by calcitriol in primary brown fat cultures [19]. Therefore, it is quite possible that vitamin D may enhance the intestinal mMDH activity through inhibition of redox-mediated transcriptional up-regulation of UCP-2, thereby reducing mitochondrial proton leak and stabilizing the mitochondrial function.

Glucose-6-P-dehydrogenase, which catalyzes NADPH synthesis, is another target of vitamin D in the intestine. The activity of this enzyme in the middle part of the duodenum villus is 60% lower in vitamin D-deficient rats than in normal rats. Intraperitoneal injection of calcitriol to vitamin D-deficient animals increased G6PD activity within 30 min, attaining normal levels within 2 h [34]. Bao et al. [35] demonstrated that calcitriol protects nonmalignant human prostate epithelial cells from oxidative stress by elimination of ROS-induced cellular injuries by transcriptional activation of G6PD. Therefore, it is quite possible that another mechanism by which calcitriol enhances the intestinal mMDH activity would be through restoring the proper redox environment by activation of G6PD, which is also considered a key antioxidant enzyme [35].

Due to the fact that calcitriol not only enhances mMDH activity, but also the catalysis of all NAD-dependent oxidoreductases from the TCA cycle [8], the molecular mechanisms triggered by calcitriol to increase mMDH activity might also occur with the other NAD-oxidoreductases of the TCA cycle. Consequently, the flux rate of electrons to be funneled through the electron respiratory chain would be increased, and so does ATP production. These molecular events would stimulate the intestinal transcellular Ca^{2+} pathway because the entrance of Ca^{2+} ions into the enterocytes promoted by calcitriol would be followed by a rapid exit through the Ca^{2+} pump and the Na^+/Ca^{2+} exchanger, which are motorized directly and indirectly by the ATP hydrolysis, respectively.

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