

## EFFECT OF VITAMIN D ON CARBONIC ANHYDRASE ACTIVITY EXPERIMENTAL RESEARCH *IN VITRO* AND *IN VIVO*

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### Abstract

The purpose of this study was to determine the effect of vitamin D on the activity of erythrocyte carbonic anhydrase isoenzymes (CA) *in vitro* and *in vivo*. *In vitro*, the effect was assessed by purified CA I and CA II isoenzymes using vitamin solutions with concentrations between  $10^{-8}$  and  $10^{-4}$  M, and *in vivo*, in mice erythrocytes from Male Sprague-Dawley breed. Measurement of enzyme activity was performed by the hydration reaction of CO<sub>2</sub> (Stopped-flow method). *In vitro* there is a direct inhibition of CA by a dose-response relationship maximum at concentration  $10^{-4}$  M. *In vivo*, the inhibitory effect is stronger for vitamin D<sub>3</sub>. Both *in vitro* and *in vivo*, vitamins D<sub>2</sub> and D<sub>3</sub> produced a significant decrease in CA II activity compared to CA I.

### Rezumat

Scopul acestui studiu a fost de a determina efectul vitaminelor D asupra activității izoenzimelor anhidrazei carbonice eritrocitare (AC) *in vitro* și *in vivo*. *In vitro*, efectul a fost urmărit pe izoenzimele AC I și AC II purificate folosind soluții de vitamine cu concentrații cuprinse între  $10^{-8}$  și  $10^{-4}$  M, iar *in vivo* pe eritrocite provenite de la șoareci din rasa Male Sprague-Dawley. Măsurarea activității enzimatică s-a făcut prin urmărirea reacției de hidratare a CO<sub>2</sub> (metoda *Stopped-flow*). *In vitro* se observă o inhibiție directă a AC după relația doză-răspuns, maximă la concentrația  $10^{-4}$  M. *In vivo* efectul inhibitor este mai puternic pentru vitamina D<sub>3</sub>. Atât *in vitro*, cât și *in vivo*, vitaminele D<sub>2</sub> și D<sub>3</sub> produc o scădere semnificativă a activității AC II comparativ cu AC I.

**Keywords:** Carbonic anhydrase, vitamin D, inhibition

### Introduction

Carbonic anhydrase (CA, EC 4.2.1.1.) is an enzyme found in most organs of the human body as different isoenzymes that catalyze reversible the hydration of CO<sub>2</sub> to bicarbonate (HCO<sub>3</sub><sup>-</sup>) at physiological pH [10, 12, 15, 22]. The enzyme participates in a variety of physiological processes involving pH regulation, CO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> transport, ion transport and maintenance of

hydroelectrolytic balance [5]. Evidence suggests that the enzyme could be involved in transmitting signals at the molecular level, cell growth and bone resorption and possibly in oncogenesis and cancer [2].

Many of the CA isoenzymes involved in these processes may represent important therapeutic targets for treating several conditions: edema, hypertension, glaucoma, obesity, cancer, epilepsy and osteoporosis [21].

An increased awareness of osteoporosis (a disease characterized by deterioration of the skeletal system architecture due to decreased bone formation or increased bone resorption) has intensified the concerns of researchers to elucidate the pathogenic mechanisms involved in its appearance [3]. Deficiency of vitamin D, followed by a decrease in serum calcium leads to secondary hyperparathyroidism causing an increased bone resorption [9, 20]. In addition, an increased level of parathyroid hormone (PTH) stimulates the activity of carbonic anhydrase, especially the CA II isoenzyme located in the osteoclasts, which is involved in bone resorption [14].

Bone metabolism can be assessed by measuring bone markers in serum or urine [8]. Osteocalcin is, besides collagen, a major protein of the bones, involved mainly in the bone mineralization and calcium homeostasis [6]. Osteocalcin synthesis is modulated by vitamins K and D. The level of osteocalcin can be an excellent marker to assess long-term effects of antiresorptive treatment [7].

Vitamins D ( $D_2$  and  $D_3$ ) are involved in maintaining calcium homeostasis and modulate many physiological and pathological processes including bone resorption [9].

The purpose of our research was to determine the effect and mechanism of action of vitamin D on the activity of CA isoenzymes *in vitro* and *in vivo*.

## Materials and Methods

### *In vitro* study

To determine the effect of vitamins D ( $D_2$  and  $D_3$ ) on the purified CA I and CA II, the study was accomplished by monitoring the dose-response relationship at concentrations between  $10^{-8}$  and  $10^{-4}$  M.

Reagents (pure substances) were purchased from the company SIGMA Deisenhofen, Germany. Stock solutions were prepared at the concentration of  $10^{-3}$  M, by weighing and dissolving in distilled water, and adjusting to a pH of 7.5. For each substance there were made successive dilutions, obtaining concentrations between  $10^{-4}$  M and  $10^{-8}$  M.

CA activity was assayed by *Stopped-flow* [17] method following the hydration reaction of  $CO_2$  with a rapid kinetic spectrophotometer model SF-51 HI-TECH MX (England).

Basal activity of purified CA I and CA II was measured by determining the time required for CO<sub>2</sub> hydration reaction in the presence of the enzyme. Enzyme activity expressed as enzyme units (EU/mL) was calculated using the equation  $t_0 - t_1/t_1$ , where  $t_0$  and  $t_1$  are the times for pH change (from 7.5 to 6.5) of the nonenzymatic and the enzymatic reaction, respectively.

In the next phase of the experiment to each isoenzyme of CA there were added increasing concentrations of vitamins and enzymatic activities were measured. It has been calculated the percentage of activation or inhibition of CA I and CA II, for each vitamin separately.

#### ***In vivo study***

The study protocol was approved by the Ethics Committee of the Research and Nursing Center, Șimleul Silvaniei, Sălaj, Romania. All procedures were carried out in accordance with the Directive 86/609/EEC of 24th November 1986, regarding the protection of animals used for experimental and other scientific purposes.

Sixty male rats, Male Sprague-Dawley breed, weighing 300-400g were used in the study. They were kept under standard conditions, in an isolated room with a constant temperature of 20°C, with free access to water, and fed with standard laboratory chow. They were randomly divided into 3 groups (20 rats per group) and placed in separate cages during the study. Solutions of substances to be analyzed were injected intravenously into the jugular vein through a PE-50 cannula type, as follows:

- Group 1 - vitamin D<sub>2</sub>, i.v., 800.000 IU;
- Group 2 - vitamin D<sub>3</sub>, i.v., 40.000 IU;
- Group 3 (Control group) - 0.9% NaCl isotonic solution.

Prior to administration of substances and one hour after administration, blood was collected and the activity of CA I and II from the erythrocyte hemolysate was measured. The enzyme activity expressed as enzyme units (EU/mL) was calculated using the equation  $t_0 - t_E/t_E$ , where  $t_0$  and  $t_E$  are the times for pH change (from 7.5 to 6.5) of the nonenzymatic and the enzymatic reaction catalyzed by erythrocyte CA, respectively. Differentiation of CA I from CA II activity was performed using Nicosilvanil Test [17].

#### ***Statistical Analysis***

Changes of enzyme activity are presented as mean  $\pm$  standard deviation. For statistical processing of data we used the Student *t* test. The level of statistical significance was set at  $p < 0.05$ .

## Results and Discussion

### Results for *in vitro* study

In table I is presented the progressive decrease of the activity of purified CA I and CA II isoenzymes after the addition of vitamins D<sub>2</sub> and D<sub>3</sub>.

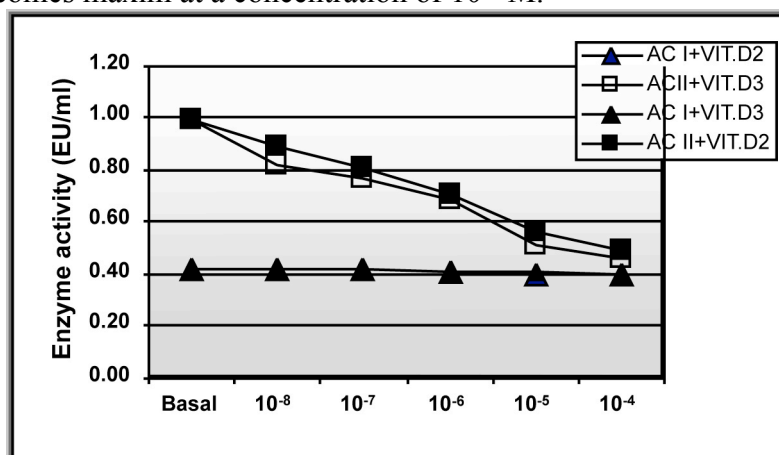
**Table I**

Effect of vitamin D on CA isoenzymes

Substance	Conc. (M)	Pure CA I Basal= $0.425 \pm 0.01$ (EU/mL)	Pure CA II Basal = $1.00 \pm 0.06$ (EU/mL)
Vitamin D <sub>2</sub>	$10^{-8}$	$0.421 \pm 0.01$	$0.891 \pm 0.01$ *
	$10^{-7}$	$0.417 \pm 0.02$	$0.811 \pm 0.01$ *
	$10^{-6}$	$0.409 \pm 0.03$	$0.706 \pm 0.02$ *
	$10^{-5}$	$0.403 \pm 0.01$	$0.561 \pm 0.01$ *
	$10^{-4}$	$0.401 \pm 0.02$	$0.492 \pm 0.03$ *
Vitamin D <sub>3</sub>	$10^{-8}$	$0.424 \pm 0.01$	$0.823 \pm 0.01$ *
	$10^{-7}$	$0.421 \pm 0.02$	$0.774 \pm 0.01$ *
	$10^{-6}$	$0.415 \pm 0.01$	$0.685 \pm 0.02$ *
	$10^{-5}$	$0.408 \pm 0.01$	$0.514 \pm 0.01$ *
	$10^{-4}$	$0.404 \pm 0.02$	$0.463 \pm 0.02$ *

\*p<0.05 compared with the control group

The decrease of enzyme activity depends on the inhibitor concentration (Figure 1). The effect is present at the concentration of  $10^{-8}$  M and becomes maxim at a concentration of  $10^{-4}$  M.



**Figure 1**

The *in vitro* effect of vitamin D on CA isoenzymes

Table II shows the percentage of inhibition produced by vitamin D on CA isoenzymes activity. The percentage is expressed with the sign (-) to highlight that it is an inhibition, therefore a decrease in the enzymatic activity under the basal value.

**Table II**

The percentage of inhibition produced by vitamin D  
on CA isoenzymes activity

Substance	Conc. (M)	Pure CA I	Pure CA II
Vitamin D <sub>2</sub>	10 <sup>-8</sup>	- 1%	- 11 %
	10 <sup>-6</sup>	- 3%	- 29 %
	10 <sup>-4</sup>	- 5%	- 51 %
Vitamin D <sub>3</sub>	10 <sup>-8</sup>	- 1 %	- 18 %
	10 <sup>-6</sup>	- 2 %	- 32 %
	10 <sup>-4</sup>	- 4 %	- 54 %

From the study on dose-response relationship it resulted that vitamin D used for *in vitro* experiments produced a direct inhibition of CA II isoenzyme. The effect occurs at concentrations of 10<sup>-8</sup> M and increased progressively, with increasing the inhibitor concentration, reaching the maximum peak at the concentration of 10<sup>-4</sup> M. Studies were not performed at concentrations greater than 10<sup>-4</sup> M because this concentration is equivalent to pharmacological doses.

Analyzing the results for each type of isoenzyme it can be seen that the inhibitory effect is present on the pure CA II, while the effect on pure CA I is insignificant.

#### Results for *in vivo* study

CA I activity in red blood cells in the Control group was 0.335 ± 0.010 EU/mL and CA II activity was 1.198 ± 0.102 EU/mL. For both groups that received the treatment with vitamin D, the activity of CA II from erythrocyte decreased significantly (p < 0.001), while CA I activity did not record significant changes compared with Control group values.

Values for CA I and CA II from erythrocyte are presented in Table III:

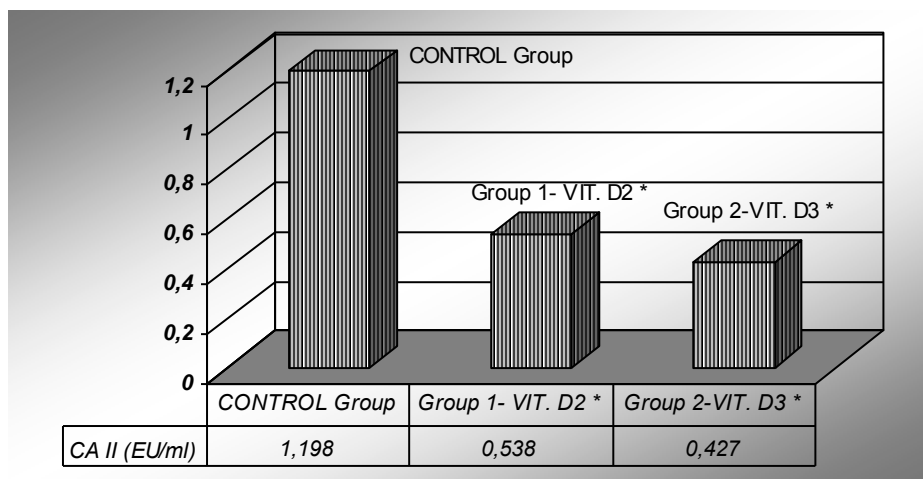
**Table III**

Enzymology results in experimental animals treated with  
vitamin D, compared with the Control group

Group	Treatment	Erythrocyte CA I (EU/mL)	Erythrocyte CA II (EU/mL)
1	Vitamin D <sub>2</sub>	0.324±0.006	0.538±0.019*
2	Vitamin D <sub>3</sub>	0.319±0.002	0.427±0.015*
3	Control group	0.335±0.010	1.198±0.102

\*p<0.05 compared with the control group

In Figure 2 are shown the inhibitory effects of vitamin D on erythrocyte CA II activity.



\*p<0.05 compared with the control group

**Figure 2**  
Changes of erythrocyte CA II activity at rats  
treated with vitamin D

Both studies show significant changes in the activity of CA II, the most widespread isoenzyme of carbonic anhydrase gene family, virtually present in all human organs or tissues. CA II is present in osteoclasts at similar concentrations as those in kidney and erythrocytes [21]. In 2002, Roussel *et al.* in a study that defined the main biochemical mechanisms involved in bone resorption indicated that CA II provides the proton source for extracellular acidification prior to dissolution of bone matrix [18], and Shinohara *et al.* showed that acetazolamide (AZ), an inhibitor of CA II, influenced the differentiation and osteoclast activity, reducing bone resorption [19]. Moreover, the inhibitory effect of thiazides, used as antihypertensive drugs, on CA II activity was described by many authors [4, 13, 16]. The effect of thiazide diuretics on bone may be explained by reducing urinary calcium excretion and partial suppression of PTH secretion, followed by decreased bone remodeling [11]. Similarly, the beneficial effects of vitamin D administration on bone, determined by the restoring of calcium level and suppression of secondary hyperparathyroidism [1] could be produced through inhibition of CA II activity by vitamin D, inhibition which in our studies, both *in vitro* and *in vivo*, occurs directly after a dose-response relationship.

### Conclusions

These studies reveal that vitamin D inhibits CA activity, both *in vitro* and *in vivo*. The inhibitory effect is predominantly on CA II, involved in the process of bone resorption, while the effect on CA I is insignificant. *In vitro* there is a direct inhibition of CA by a dose-response relationship, maximum at the concentration of  $10^{-4}$  M. *In vivo*, the inhibitory effect is stronger for vitamin D<sub>3</sub>.

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