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EFFECT OF VITAMIN E ON CARBONIC ANHYDRASE AND SUPEROXIDE DISMUTASE 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 E on erythrocyte carbonic anhydrase (CA) and superoxide dismutase (SOD) activity in vitro and in vivo. In vitro effect was followed by purified isoenzymes CA I and CA II using vitamin solutions with concentrations between 10^{-8} and 10^{-4} M and in vivo in mice erythrocytes from Male Sprague-Dawley breed. Groups of rats were given vitamin E (75 mg/kg/day, i.g.), 3,4-Benzpyren (200 ppm/day, i.g.), Vitamin E + 3,4-Benzpyren (75 mg/kg/day + 200 ppm/day, i.g), and a Control group only Placebo. Measurement of enzymes activity was done by following the hydration reaction of CO_2 (stopped-flow method) for CA, respectively the enzymatic inhibition of oxidation of epinephrine to adrenochrome for SOD. In vitro there is a direct activation of CA by dose-response relationship maximum at concentration 10^{-4} M. Both in vitro and in vivo, vitamin E produced a increase in CA I and CA II activity, and the effect is stronger on CA II isoenzyme. In vivo studies show that vitamin E decreases CA and SOD inhibition caused by Benzpyren.
Keywords: vitamin, carbonic anhydrase, superoxide dismutase, activity

INTRODUCTION

Carbonic anhydrases (CAs) are zinc metalloenzymes that catalyze the reversible hydration of CO_2 and are major player in many physiological and pathological processes, including renal and male reproductive tract acidification, formation of gastric acid, respiration and transport of CO_2 and bicarbonate between metabolizing tissues and lungs, bone resorption, biosynthetic reactions (gluconeogenesis, lipogenesis, ureagenesis), signal transduction, cell proliferation and oncogenesis (Breton S., 2008; Supuran C.T., 2008).

Many of the CA isoenzymes involved in these processes may represent important therapeutic targets to treat several conditions: edema, hypertension, glaucoma, obesity, epilepsy, osteoporosis and cancer (Gilmour K.M., 2010).

CAs are involved in cellular pH regulation and have been implicated in some pathogenic processes including tumour progression. As major enzymes involved in pH regulation, CAs are needed to maintain intratumoural acid-base homeostasis. Of the 13 active CA isozymes, at least CAII, which is down-regulated in most cancers, CAIX and CAXII are expressed in various tumours and represent promising targets for novel anticancer therapies (Parkkila S., 2008).

SOD is one of the most effective intracellular enzymatic antioxidants catalyzes the dismutation of superoxide radical $O_2^{\bullet -}$ to O_2 and to the less-reactive species, hydrogen peroxide (H_2O_2) (Kataria et al., 2010). By scavenging $O_2^{\bullet -}$, SOD form the first line of defence against oxidative stress and its subsequent effects (Landis et al., 2005). Superoxide dismutase exists in several isoforms, differing in the nature of the active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features. In humans there are three forms of SOD: cytosolic (Cu, Zn-SOD), mitochondrial (Mn-SOD), and extracellular SOD (EC-SOD)(McCord et al.,1969; Zelko et al., 2002) Disturbances in the functioning of the SOD isoforms lead to numerous pathological changes in the human organism, including tumor disease (Johnson et al., 2005). For certain tumour cells the activity of total SOD (Cu, Zn-SOD and Mn-SOD) has been found to be reduced. The redox status of cancer cells has a great influence on tumor progression. SOD isoenzymes are one of the main controllers of the redox state, therefore therapies affecting SOD activity or expression in cancers could aid in elimination of cancer cells (Skrzycki et al., 2009).

Vitamin E is a family of eight compounds with α-tocopherol being the predominant form in mammals. Due to its antioxidant properties vitamin E has been classified as the major lipid-soluble antioxidant which protects lipids and membranes from oxidative damage in vitro and in vivo (Brigelius-Flohe et al., 2002) and its implicated in prevention or delayed progression of diseases believed to be caused or aggravated by oxidative stress (Mehrad and Sudagar, 2010), such as cardiovascular disease, cancer or neurodegenerative symptoms (Lonn et al., 2005).

Benzpyren (polyaromatic hydrocarbons) is ubiquitous in the environment, being intensely studied for toxic effects in laboratory animals and human populations. Benzpyren toxicity is often mediated by oxidative metabolism to reactive intermediates that interact with

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macromolecules leading to alterations in target cell structure and function. More recent evidence suggests that disruption of cellular signaling pathways involved in the regulation of growth and differentiation contribute significantly to the toxicity of Benzpyren at the molecular level and may lead to carcinogenesis, atherogenesis and teratogenicity (Miller et al., 2001).

Because exposure to carcinogens cause an inhibition of CA activity and a marked generation of ROS in tissues, with the initiation of oxidative damage that contributes to the onset of local carcinogenesis process, in our study we evaluated the effects of Benzpyren, vitamin E and their association on CA and SOD activity and we attempt an explanation of the biochemical mechanism by which this vitamin could be involved in antitumor and antioxidant protection.

MATERIALS AND METHODS

In vitro study

To determine the effect of vitamin E 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 concentration of 10^{-3} M, by weighing and dissolving in distilled water, and adjusted to a pH of 7,5. For each substance were made then successive dilutions, obtaining concentrations between 10^{-4} M and 10^{-8} M.

CA activity was assayed by Stopped-flow method following the hydration reaction of CO_2 (Khalifah R.G., 1971) 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_2 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 were added increasing concentrations of vitamin and enzymatic activities were measured. It has been calculated the percentage of activation or inhibition of CA I and CA II for each concentration of vitamin separately.

In vivo study

Based on in vitro results, that show an activator effect of vitamin E on CA isoenzymes, we also conducted an experimental study in laboratory animals in which we explore the new aspects of the relationship between vitamin E and CA and SOD activity.

Study protocol was approved by the Ethics Committee of the Research and Nursing Center, Şimleul Silvaniei, Sălaj.

In this study we followed the effect of carcinogens in experimental animals (alone and in combination with vitamin E) on CA isoenzymes and SOD activity.

Forty male rats from Male Sprague-Dawley breed, weighing 170±20g were used in the study. During the study the animals were kept under standard conditions, in isolated room with constant temperature of 23±2°C, with constant access to water, but fed with standard laboratory chow, the same for all and a day-night circadian cycle of 12 hours. They were randomly divided into 4 groups (10 individuals each) and placed in separate cages during the study. They were treated by gavage for 10 days as follows:

- Group 1 - Control group that received Placebo
- Group 2 - Vitamin E, 75 mg/kg/day
- Group 3 - 3,4 -Benzpyren, 200 ppm/day
- Group 4 - Vitamin E 75 mg/kg/day + 3,4 -Benzpyren 200 ppm/day

Blood samples have been taken after 10 days and the activity of CA I, CA II and SOD from erythrocyte hemolysate was measured. For CA, 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 done using Nicosilvanil Test (Puscas et al, 1999).

SOD activity was assessed according to the method that follows enzymatic inhibition of oxidation of epinephrine to adrenochrome (Misra et al., 1972) with a rapid kinetic spectrophotometer model SF-51MX HI-TECH (Hi-Tech Scientific Ltd. Salisbury, England) at a wavelength of 480 nm and the results are expressed as enzyme units (EU).

Changes of enzyme activity are presented as mean ± standard deviation.

For statistical processing of data we used the Student's test. The level of statistical significance was set at $p < 0.05$.

RESULTS AND DISCUSSIONS

Results for in vitro study

In Table I is presented progressive increase of purified CA I and CA II isoenzymes activity after the addition of vitamin E.

Table I. Effect of vitamin E on CA isoenzymes
Values are presented as mean ± standard deviation; n = 5 measurements for each concentration;
*Significant difference (p <0.05) compared with basal activity of the enzyme values (Student's test).

Substance	Conc. (M)	**Pure CA I** ***Basal*= *0.425 ± 0.01* (UE/ml)**	**Pure CA II** ***Basal* = *1.00±0.01* (UE/ml)**
	10^{-8}	0.472 ± 0.01 *	1.181 ± 0.02 *
	10^{-7}	0.497 ± 0.02 *	1.263 ± 0.03 *
Vitamin E	10^{-6}	0.531 ± 0.01 *	1.329 ± 0.02 *
	10^{-5}	0.569 ± 0.03 *	1.447 ± 0.01 *
	10^{-4}	0.608 ± 0.02 *	1.572 ± 0.01 *

Fig. 1 shows the effect of vitamin E on CA isoenzymes activity, effect that depends of concentration of vitamin. There is an effect showing at concentration of 10^{-8} M that reaches its peak at 10^{-4} M.

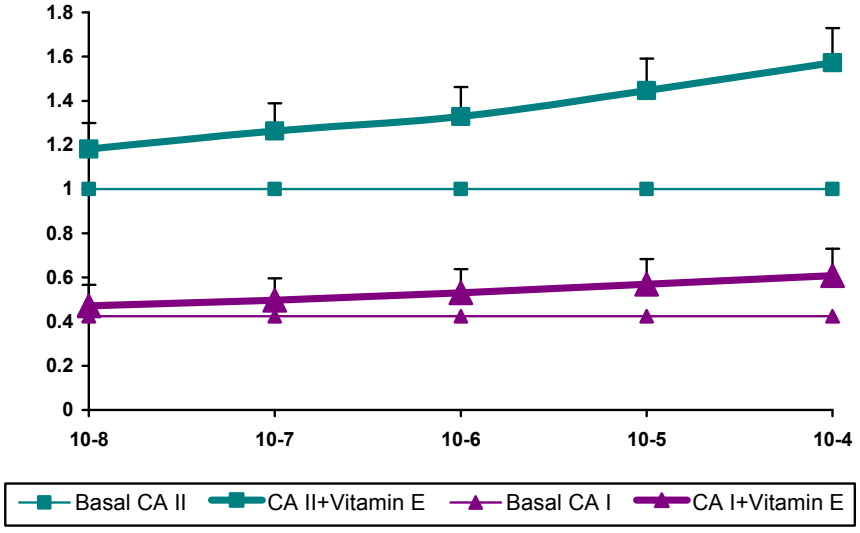

Fig. 1 In vitro effect of vitamin E on CA isoenzymes activity

From study on dose-response relationship resulted that vitamin E used for in vitro experiments produce a direct activation of CA I and CA II isoenzymes. The effect occurs at concentrations of 10^{-8} M and increased progressively, with increasing activator concentration, reaching maximum peak at concentration of 10^{-4} M. Studies were not performed at concentrations greater than 10^{-4} M vitamin because of this concentration is covered pharmacological doses and the results obtained at these levels are not conclusive for physiological processes.

Vitamin E produces an activation of 57% on pure CA II isoenzyme activity, while activation determined on pure CA I isoenzyme activity at the same concentration is 43%.

Results for in vivo study

Erythrocyte CA I activity in the Control group was 0.094±0.327 EU/ml and erythrocyte CA II activity was 0.143±1.271 EU/ml. Results for groups that received vitamin E, 3,4 -Benzpyren and their association are presented in Table II, Fig. 2 and Fig. 3.

Table II Enzymology results for CA at experimental animals treated with vitamin E, 3,4-Benzpyren and their association compared with the Control group
*statistically significant difference compared with Control group (p<0.05)

Group	Treatment	Erythrocyte CA I (EU/ml)	Erythrocyte CA II (EU/ml)
1.	**Control**	0,327 ± 0,094	1,271 ± 0,143
2.	**Vitamin E**	0,468 ± 0,123	2,158 ± 0,224*
3.	**3,4-Benzpyren**	0,154 ± 0,069	0,192 ± 0,077*
4.	**Vitamin E + 3,4-Benzpyren**	0,413 ± 0,112	1,995 ± 0,136*

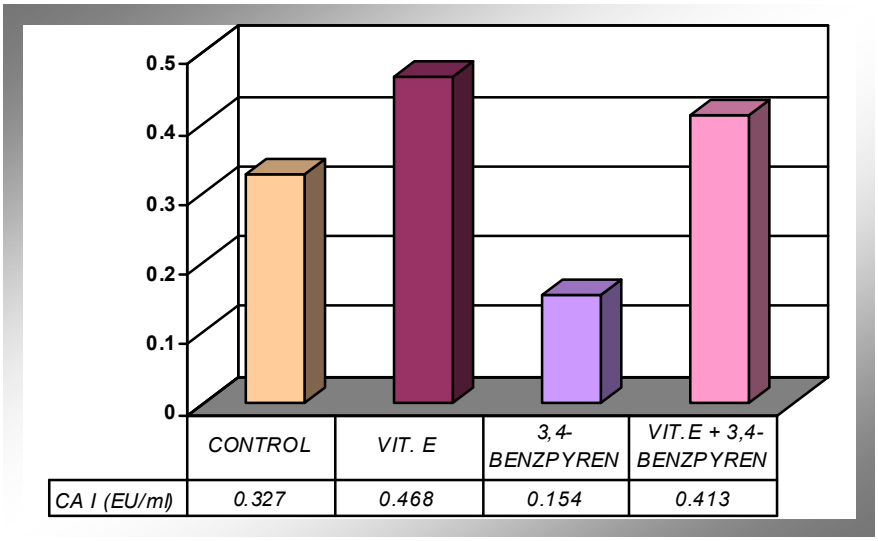

Fig. 2 Changes in erythrocyte CA I activity at experimental animals treated with Vitamin E, 3,4-Benzpyren and their association, compared with the Control group

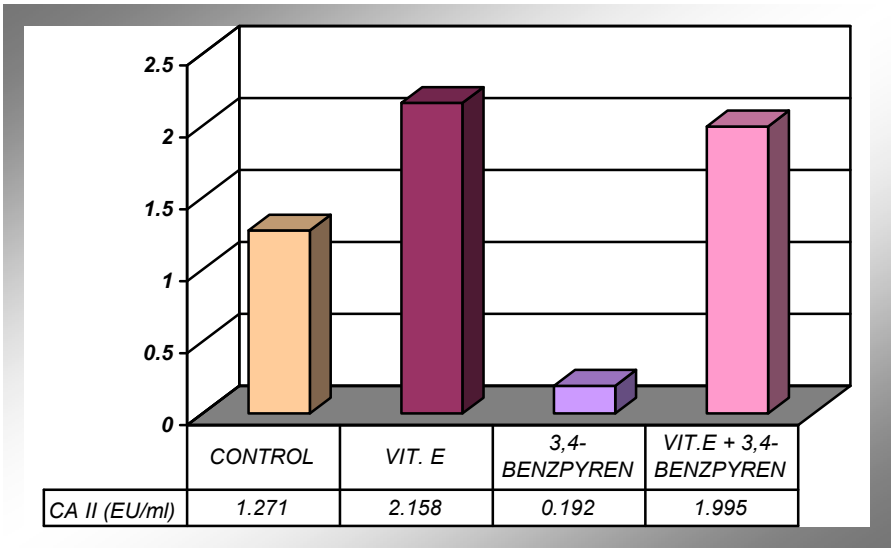

Fig. 3 Changes in erythrocyte CA II activity at experimental animals treated with Vitamin E, 3,4-Benzpyren and their association, compared with the Control group

The results reveal that Vitamin E administered as monotherapy increases the activity of CA I and II, and the effect is stronger on CA II isoenzyme.

3,4 -Benzpyren produces a strong decrease of CA isoenzymes, reaching over 85% inhibition of CA II, isoenzymes involved in carcinogenesis.

The association Vitamin E + 3,4 -Benzpyren completely antagonize the inhibitory effect of carcinogenic compounds on CA isozymes, even producing their activation, but lower than that achieved by vitamin E administered as monotherapy. In a study conducted in 2005, Çiftçi et al. states that nicotine markedly inhibited CA activity in heart, lung, stomach, and liver tissues but this inhibitory effect was totally eliminated in lung and was attenuated in heart, stomach, and liver tissues in the nicotine + vitamin E group (Çiftçi et al., 2005), our findings being in accordance with these.

The research team led by prof. Puşcaş demonstrated that the action of carcinogens, related to inhibition of CA, mostly of CA II and CA IV, followed by alkalinisation of intracellular pH, might be held responsible for the carcinogenic effect of these substances (Puşcaş et al., 1995). Without being able to deny the beneficial role of vitamin E in many metabolic processes, a possible antitumor mechanism would be linked to activation of CA, with decreasing of intracellular pH, which is

unfavourable for carcinogenesis, thus explaining its antitumor properties.

With regard to SOD activity, in the Control group, its value was 6.23±0.29 EU. Results for groups that received Vitamin E, 3,4 -Benzpyren and their association are presented in Table III and Fig. 4.

Table III Enzymology results for SOD at experimental animals treated with vitamin E, 3,4-Benzpyren and their association compared with the Control group

Group	Treatment	SOD activity (EU/ml)
1.	**Control**	6.23±0.29
2.	**Vitamin E**	7,82±0,34
3.	**3,4-Benzpyren**	3,56±0,63
4.	**Vitamin E + 3,4-Benzpyren**	5,14±0,47

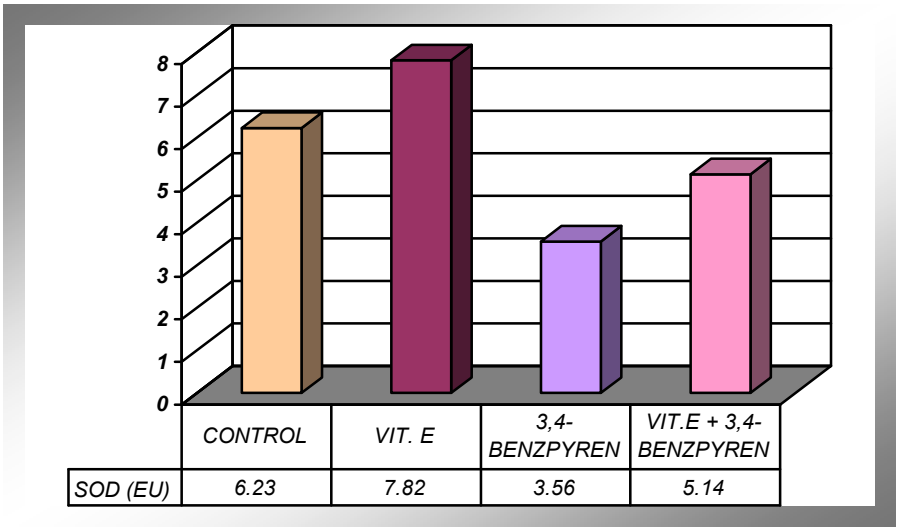

Fig. 4 Changes in erythrocyte SOD activity at experimental animals treated with Vitamin E, 3,4-Benzpyren and their association, compared with the Control group

The study shows a slight increase of SOD activity in the group receiving vitamin E as monotherapy. For group who received 3,4 -Benzpyren, a substance known to have a powerful carcinogenic potential, SOD activity was decreased by approximately 57%, probably due to modification of antioxidant systems caused by reactive oxygen species that result from chronic exposure to carcinogenic action (Kim et al., 2000). For group that received Vitamin E + 3,4 -Benzpyren association SOD activity reduction is not so marked as in Group 3. This can be explained by the fact that concomitant administration of antioxidants should be able to spare the endogenous antioxidant systems, conclusion that is in accordance with other research (Kadkhodaee et al., 2007, Kashani et al., 2010).

CONCLUSIONS

These studies reveal that vitamin E increases CA activity, both in vitro and in vivo, and the effect is stronger on CA II isoenzyme. In vitro there is a direct activation of CA by dose-response relationship, maximum at concentration of 10-4 M. In vivo, 3,4 -Benzpyren markedly inhibited CA activity but this inhibitory effect was completely antagonize by vitamin E. In vivo, vitamin E cause a slight increase of SOD activity and 3,4 –Benzpyren cause a decrease in SOD activity by approximately 57%. Concomitant administration of vitamin E partially attenuated the inhibitory effect of Benzpyren at group that received Vitamin E + 3,4 -Benzpyren association.

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