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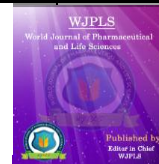

1-(CARBOXY)-1-(N-ACETYLAMINO)-2-(3, 5'-DI- TRET -BUTYL-4'-HYDROXYPHENYL-PROPIONATE SODIUM PREVENTS OF OXIDATIVE STRESS IN ANIMALS AND PLANTS

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ABSTRACT

In stress conditions, the formation of ROS by mitochondria increases, which leads to a shift in the antioxidant - prooxidant balance toward increasing the ROS content in the cell. The interaction of ROS with polyunsaturated fatty acids, which enter into the composition of the lipids of mitochondrial membranes, leads to the activation of lipid peroxidation (LPO). Herewith in 2.5 - 3 times increased fluorescence intensity of LPO products in the membranes of these organelles. Spatial-obstructed phenols, in particular, 1-(carboxyl)-1-(N-acetylamino)-2-(3',5'-di-tret-butyl-4'-hydroxyphenyl)-propionate sodium (sodium anphen) decrease the LPO intensity to control values and increases the activity of the I complex of the mitochondrial respiratory chain, providing high functional activity of these organelles. The prevention of mitochondrial dysfunction is associated with an increase in the resistance of plant and animal organisms to stress factors.

KEYWORDS: ROS, mitochondria, lipid peroxidation, I complex of the mitochondrial respiratory chain.

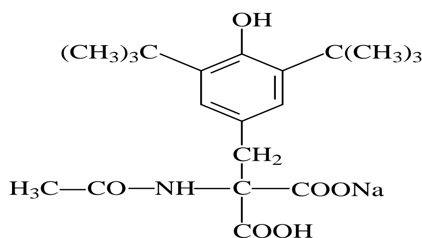
INTRODUCTION

Mitochondria, as an energy metabolism regulator, play one of the basic roles in the organism response to the action of stressors.[1] Approximately 1-3% of oxygen consumed by mitochondria as a result of reduction with 1 -2 electron form active forms of oxygen (ROS), which participate in the cellular redox signaling. Normally, the stationary level of ROS in organs and tissues is rather low (on the order of 10^{-10} - 10^{-11} M) due to availability the enzymatic and non-enzymatic systems of regulation of the accumulation and elimination of ROS. In conditions of stress, the formation of ROS by mitochondria is enhanced to a greater or lesser extent, which leads to a shift of antioxidant - prooxidant balance in the direction of increasing the ROS content in the cell. Excessive production of ROS by electron-transport chains, first of all, can lead to damage to mitochondrial DNA (mtDNA), which unlike nuclear DNA is not protected by histones.[2,3] As a result of mutations of mtDNA, oxidative damage of proteins and activation of lipid peroxidation, there may be a disruption in the energy supply of cells, which ultimately leads to pathological changes.[3,4,5,6] In this regard, it could be assumed that drugs that increase the body's resistance to stress factors (adaptogens) should influence the generation of ROS by these organelles. Antioxidants primarily have a claim on this role. Effective interceptors of radicals are phenolic antioxidants, including spatially hindered phenols.[7] The antioxidant properties of phenols are associated with the presence in their structure of weak phenolic hydroxyl groups, which easily give up their hydrogen atom when interacting with free radicals. In this case, the phenols act as traps of free radicals, turning themselves into low-activity phenoxyl radicals. Note that the introduction of synthetic antioxidants into the body has a significant influence not only on the processes of lipid peroxidation, but also on the metabolism of natural antioxidants. The action of natural and synthetic antioxidants may sum up, resulting in an increase in the effectiveness of their protective action. In addition, the introduction of synthetic antioxidants can influence the synthesis and utilization of natural antioxidants, and therefore, cause changes in the antioxidant activity of lipids.[8] Thus, synthetic antioxidants can be used in biology and medicine as preparations that affect not only the processes of free radical oxidation, but also the system of natural antioxidants, that affecting the change in antioxidant activity..

The effectiveness of antioxidants depends on the dose of the drugs is non-linearly. In high concentrations,

antioxidants are pro-oxidants, i.e. they do not inhibit, but activate free radical reactions.[9] Therefore, for antioxidants, it is necessary to select the most effective concentrations for increasing resistance of both animal and plant organisms to stress factors.

In connection with this regard, the object of research was chosen a drug which is a spatially-hindered phenol of sodium anphen (1- (carboxy) -1- (N-acetylamino) -2- (3 ', 5'-di-tret-butyl -4'-hydroxyphenyl) - propionate sodium):



Sodium Anphen

Since the respiratory chain of mitochondria of plants and animals has a common pattern of organization, and the principal differences relate to the CN-resistant electron transfer and the structure of the NADH-dehydrogenase region of the respiratory chain,[10] in connection with this the basic mechanisms of antioxidants actions in the capacity of adaptogens were studied in both as on the rat liver mitochondria thus and on mitochondria of etiolated pea seedlings.

The aim of the study was also to study the functional state of rat liver mitochondria under stress conditions and the effect on it of the study drug in a wide range of concentrations. In addition, the effect of sodium anphen on the bioenergetic characteristics of mitochondria of 6-day etiolated pea seedlings (*Pisum sativum* L), variety Alpha, was studied.

MATERIAL AND METHODS

The experiment was performed in the Institute of biochemical physics. N. M. Emanuel Russian Academy of Sciences. Tests was performed on male rats of Wistar line weighing 120-140 g. and on pea seedlings (*P. sativum*), cultivar Alpha, which were grown in standard conditions.

Regulatory standards

The study was performed according to the Rules of laboratory practice in the Russian Federation, in accordance with the rules adopted by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (ETS 123), Strasburg, 1986). The research was performed according to the approved protocol, in accordance with standard operating procedures of the researcher (SOPR), as well as with the Guidelines at laboratory animals and alternative models in biomedical researches on laboratory animals.[11] An experimental Protocol was developed with the participation and approval of the bioethical Commission of the Institute.

Germination of Pea Seeds

The seeds were washed with soap solution and 0.01% KMnO4 solution. The control group of the seeds were soaked in water for 30 minutes, and the experimental group in - 10^{-13}M sodium anphen (ANP) solution. Thereafter, seeds of control group were transferred on moistened filter paper, where they were in the dark for 6 days. The seeds of the experimental group were transferred on a filter paper moistened with a 10^{-13} M solution of sodium anphen, where they were in the dark for 6 days. On the sixth day, mitochondria were isolated from the epicotyls of the seedlings of all the study groups.

Isolation of rat liver mitochondria was performed by differential centrifugation.[12] The first centrifugation at 600 g for 10 minutes, the second at 9000 g, 10 min. The pellet was re-suspended in the medium, containing: 0.25M sucrose, 10 mm HEPES, pH 7, 4.

Isolation of pea seedling mitochondria

Isolation of mitochondria from 6-day-old epicotyl of pea seedlings (*P. sativum*) were performed by differential centrifugation (at 25,000 g for 5 min and at 3000 g for 3 min).[13] Precipitation of the mitochondria was carried out for 10 min at 11000 g. The pellet was resuspended in 2-3 ml of media containing: 0.4 M sucrose, 20 mM KH2PO4 (pH 7.4), 0.1% BSA (free from fatty acids), and the mitochondria were again deposited at 11,000 g for 10 min.

Rate of Mitochondria Respiration

Respiration in mitochondria we recorded polarographically (An LP-7 polarograph, Czech Republic) using Clarke oxygen electrode. Pea sprout mitochondria were incubated in a medium containing: 0.4M sucrose, 20mM HEPES-Tris buffer (pH 7.2), 5mM KH_2PO_4, 4mM $MgCl_2$ and 0.1% BSA. The rat liver mitochondria were incubated in a medium contained 0.25M sucrose, 10mM Tris-HCl, 2mM $MgSO_4$, 2mM KH_2PO_4 and 10 mM KCl, pH 7.4 (28° C). The rate of respiration was expressed in ng-moll O_2/mg protein min.

The Level of Lipid Peroxidaion

The level of lipid peroxidation (LPO) was evaluated by the fluorescence method.[14] Lipids were extracted by the mixture of chloroform and methanol (2:1). Lipids of mitochondrial membranes (3--5 mg of protein) were extracted in the glass homogenizer for 1 min at 10ºC. Thereafter, equal volume of distilled water was added to the homogenate, and after rapid mixing the homogenate was transferred into 12-mL centrifuge tubes. Samples were centrifuged at 600g for 5min. The aliquot (3mL) of the chloroform (lower) layer was taken, 0.3mL of methanol was added, and fluorescence was

recorded in 10-mm quartz cuvettes with a spectrofluorometer (Fluoro Max Horiba Yvon, Germany). The excitation wavelength was 360nm; the emission wavelength was 420--470nm.

Protective activity of the drug was investigated using the model of acute hypobaric hypoxia.

Model of acute hypobaric hypoxia.

Modeling of acute hypobaric hypoxia in rats was carried out in the hyperbaric chamber in low-pressure atmosphere (230.40 mm Hg. tbsp.), which corresponds to the height of 9000 m above sea level. In the first minute in the chamber created the rarefaction corresponding to the 5 thousand meters (corresponding to the atmospheric pressure of 405 mm Hg. tbsp.) above sea level. In each subsequent minute "ascent" carried out on the further one thousand meters. Time staying of rats "at a height" of 9.0 thousand meters above sea level - 5.0 minutes).

To all test animals the preparation was introduced intraperitoneally at a chosen dose 45 minutes before the event.

The following reagents were used:

Methanol, chloroform (Merck, Germany), sucrose, Tris, EDTA (Ethylenediaminetetraaceticacid), FCCP (carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone), malate, glutamate, ADP, (Sigma, Aldrich, USA), Hepes (4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid) (Biochemica Ultra, for molecular biology) (MB Biomedicals, Germany).

RESULTS AND DISCUSSION

In the framework of the task to study the antistress properties of the drugs, we developed a model of "aging" (long-term storage) of mitochondria. We assumed that prolonged incubation of mitochondria in hypotonic medium at room temperature will lead to activation of the LPO. Rat liver mitochondria for activation LPO at 15 min were placed in 0.5 ml of medium containing 70mm KCl, 10 mm HEPES and 1 mm $_{KH2PO4,}$ pH 7.4., and the pea seedlings mitochondria - in 0.5 ml of medium containing 0.14 M sucrose, 10 mm HEPES and 2 mm KH_2PO_4, pH 7.4. The incubation of mitochondria in a hypotonic solution of caused a weak swelling of mitochondria and an increase in the generation of ROS, which was reflected in an increase of fluorescence intensity of LPO products in 3-4 times (Fig. 1). The introduction of sodium anphen into the mitochondrial incubation medium led to a decrease in the fluorescence of the final products of lipid peroxidation both in the membranes of mitochondria of rat liver and in the membranes of mitochondria of pea seedlings. This decrease of fluorescence intensity of LPO products had a dose dependent.

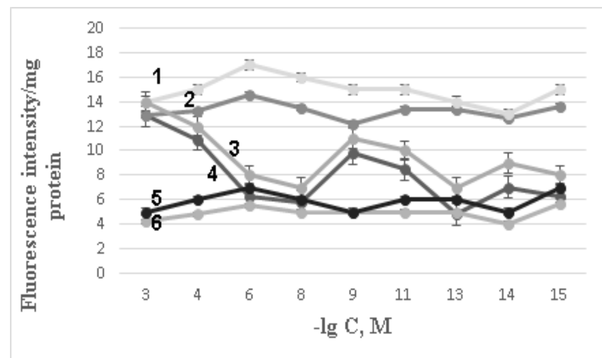


Figure 1: The effect of different concentrations of sodium anphen and "aging" on the intensity of fluorescence of LPO products. Legend: 1- «aging» of pea seedlings mitochondria; 2- «aging» of rat liver mitochondria; 3-«aging» of pea seedlings mitochondria + sodium anphen; 4-«aging» of rat liver mitochondria + sodium anphen; 5- control (pea seedlings mitochondria); 6- control (rat liver mitochondria).

The preparation in concentrations of 10^{-6}-10^{-8}, 10^{-13} and 10^{-15}M reduced the fluorescence intensity of LPO products to a control level, which indicated the antistress activity of sodium anphen, the presence of which was tested in the model of acute hypobaric hypoxia (AHH). AHH led to a 2.5-3-fold increase in the fluorescence intensity of LPO products in the membranes of rat liver mitochondria. Introduction to rats 10^{-6}M, 10^{-13}M sodium anphen 45 minutes prior to exposure prevented the activation of LPO (Fig. 2).

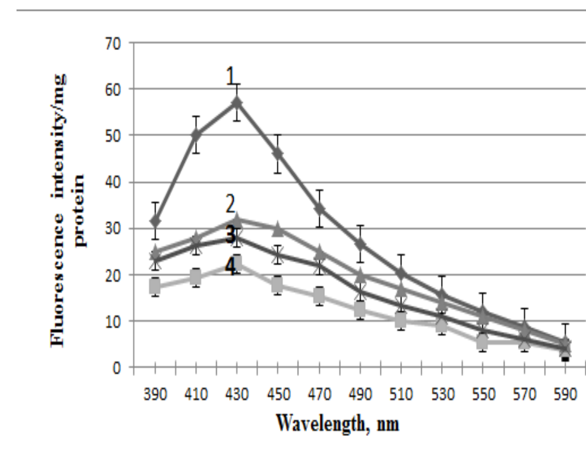


Figure 2: The effect of acute hypobaric hypoxia (AHH) and sodium anphen (ANPH) on the fluorescence intensity of the final LPO products in membranes of rat liver mitochondria.

1-AHH; 2- AHH+ 10^{-6}M sodium anphen; 3 – AHH+ 10^{-13}M sodium anphen; 4-control.

The drug caused changes in the bioenergetic characteristics of rat liver mitochondria (Table 1) and mitochondria of pea seedlings (Table 2). After 45 minutes after administration of 10^{-13}M sodium anphen to animals, the oxidation rates of NAD-dependent

substrates in the presence of ADP increased by 20%, after 3 hours they exceeded the control values by 13% and after 6 hours did not differ from the control. At the same time, the respiratory control rate increased from 2.32 ± 0.10 to 3.00 ± 0.10 (45 minutes after the injection) and to 2.81 ± 0.10 (3 hours after the injection).

Table 1: The effect of injection of 10^{-13} M anphen sodium to rats on the rate of NAD-dependent substrates oxidation by liver mitochondria, ng-mol / mg protein min (Number of -10 experiments).

Group	State 2	State 3	State 4	RCR	FCCP
Control	8.1±1.9	26.2±1.8	11,3±0.6	2.32±0,10	25.3±1.2
The time passed after the administration of sodium anphen					
45 min	5.8±2.0	31.5±2.0	10,5±0.7	3.00±0.10	32,7±2.8
3 hours	8.0±1.5	29.8±1.8	10.6±0,9	2.81±0.10	31.2±2.4
6 hours	8.6±1.9	27.0±0.9	10.7±1.3	2.52±0,10	28.5±2.0

The incubation medium contained 0.25M sucrose, 10 mM Tris-HCl, 2 mM KH_2PO_4, 5 mM $MgCl_2$ and 10 mM KCl, pH 7.5 Other additives: 1 mM malate, 4 mM glutamate, 125μM ADP, 10^{-6} M FCCP (carbonylcyanide-p-trifluoromethoxyphenylhydrazone)

It is known that under stress there is a decrease in activity of complex I of the respiratory chain of mitochondria.[15] Based on these data, we suggested that sodium anphen, by increasing the activity of the NADH dehydrogenase complex, promotes the activation of the energy metabolism of the cell, which possibly provides an increase in the resistance of the organism to stresses.

We note that the preparation increased the oxidation rate of NAD-dependent substrates by mitochondria of pea seedlings by 35% and 44% in the presence of ADP and by 52% in the presence of an uncoupler (FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone). RCR (respiratory control rate) increased by 22% (10^{-6} M sodium anphen) and 40% (10^{-13} M sodium anphen).

Table 2: The effect of sodium anphen on the rate of NAD-dependent substrate oxidation by mitochondria isolated from pea seedlings, ng-atom/mg protein min. (Number of -10 experiments).

Group	State 2	State 3	State 4	RCR	FCCP
Control	16.0±1,3	50.4±2,4	20.5±1.5	2,45±0,01	58.9±1,6
Sodium Anphen 10^{-6}M	22.5±1,4	68.4±4,6	22.8±3,4	3.00±0,02	90,0±3,2
Sodium Anphen 10^{-13}M	22.1±3,2	72.5±4,3	21.0 ±2,6	3,45±0,02	90.0±5.2

Incubation medium contained 0.4 M sucrose, 20 mM Hepes-Tris, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM EDTA, pH 7.4. Other additives: 10 mM malate + glutamate, ADP (200 μM) and FCCP (0.5 μM).

Since the mitochondria of germinating seeds are characterized by relatively low oxidation rates of NAD-dependent substrates,[16] an increase in the activity of NAD-dependent dehydrogenases helps maintain a high level of energy metabolism in the cell, which, perhaps, provides plant resistance to changing of environmental conditions. The change in the functional activity of mitochondria that affects the energy metabolism of the cell, had an impact on the physiological indices and, first of all, on the growth processes. Processing of pea seeds with 10^{-13}M sodium anphen solution stimulated growth of sprout shoots by 14% and growth of roots by 13%. (fig.3).

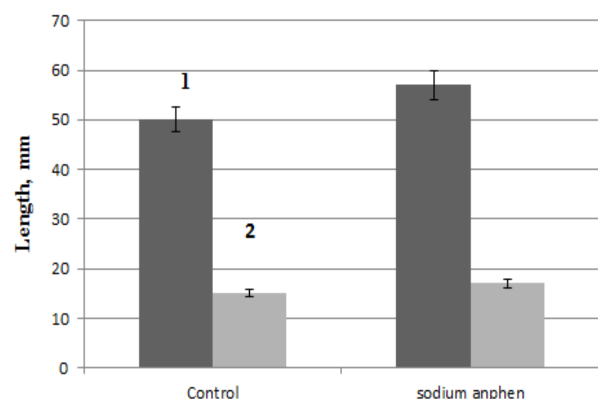


Figure 3: The effect of sodium anphen on the growth of shoots (1) and roots (2) of pea seedlings.

CONCLUSION

Based on the data obtained, it can be assumed that the protective activity of anphen sodium is due to its antioxidant properties. Reduction in the intensity of free radical oxidation processes is reflected in the low intensity of LPO. Sodium anphen prevents the activation of LPO and, apparently, contributes to the maintenance of the functional state of mitochondria. The protective properties of the preparation, apparently, are also due to its ability to activate NAD - dependent dehydrogenases.

It should be noted that the complex I of the electron transport chain of the mitochondria is the main point of arrival of the reducing agents generated in the mitochondrial matrix. Since the translocation of proton by complex I of the mitochondrial respiratory chain is associated with the oxidative phosphorylation generating ATP, an increase in the activity of NAD-dependent dehydrogenases activates the energy processes in the cell, resulting in increased resistance of the organism to changes of environmental conditions.

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