MAGNESIUM-DEPENDENT ATPASE AS A POSSIBILITY FOR THE INVESTIGATION OF DEPONATING ENVIRONMENTAL LOADS IN A HEPATOCYTE MODEL

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Abstract
Chemicals that load the environment can be chlorobenzenes with massive chemical stability. They induce a dose-dependent toxic effect in the cells of affected tissues, and because of their high frequency of occurrence in the food chain, they can be used as expositors in environmental exposure models. In this work, we wanted to investigate the magnesium-dependent ATPase activity of chlorobenzenes in hepatocytes.

Introduction
Changes in material and energy transfer can be detected in cells exposed to chlorobenzenes (ClB) [1]. ClB affect homeostasis and/or its regulation, including psycho-immuno-endocrine regulation [2]. The known effects of ClB in toxic doses have been confirmed in embryogenesis, increased risk of spontaneous abortion, intrauterine growth retardation, premature birth, and lower birth weight [3]. Furthermore, their carcinogenic, and immunotoxic effects are known [4]. However, it is very important to emphasize that these agents can also generate changes in psychological processes, such as mental and psychomotor functions [5].
Hepatocytes (HC) are significant cells in systemic relationships and xenobiotic transformations [6]. In the mentioned endodermal cells, the key of discrete energy transfer is Mg^{2+}-dependent ATPase [7]. Mg^{2+}-dependent ATPase is an integrated ectomembrane ATPase, its operation involves the hydrolysis of 3'-5'-adenosine triphosphate (ATP). These membrane units provide energy for many cellular biological processes by converting ATP molecules into 3'-5'-adenosine diphosphate (ADP) and then to 3'-5'-adenosine monophosphate (AMP) molecules, $\{P_3O_{10}^{5-}\}$ groups by generating $\{P_2O_7^{4-}\}$ and $\{PO_4^{3-}\}$ groups [8]. Hydrolysis-controlled energy changes depend significantly on the pH, temperature, and metal ion concentration of the system. In this sense, Mg^{2+} plays a key role, as they are important substrates for the formation of [Mg^{2+}-ATP] complexes, which can be further cleaved by the Mg^{2+}-dependent ATPase, and the mentioned transport process can take place [9]. The purpose of this study was to monitor the effects of different doses of ClB treatments on changes in energy transfer (Mg^{2+}-ATPase) of HC.

Experimental

In vivo protocol
Male Wistar rats (Charles River, Isaszeg, Hungary, medically certified) from different litters (weighing 120-250 g, aged 4-6 weeks at the beginning of the research) were used for cell culture

model systems. Animal care and research protocols were in full accordance with the guidelines of the University of Szeged, Hungary. During the research period, rats were kept under controlled relative air humidity of 55-65% and 22±2°C ambient temperature. The experimental animals lived under automated diurnal conditions (12 h dark and 12 h light system). Standard pellet food (CRLT/N, Charles River, Magyarország) and tap water were available *ad libitum.* Male Wistar rats were treated with combined ClB (1:1 mixture of 1,2,4- trichlorobenzene /CAS number: 2199-72-6, Sigma Aldrich, USA, St. Louis) and hexachlorobenzene / CAS number: 93952-14-8, Sigma Aldrich, USA, St. Louis), in 1 mL of 0.015% ethanol in distilled water was administered daily) in a dose of 0.1, 1.0 and 10.0 µg/b.w. kg via a gastric tube. Rats were exposed to ClB for 30 (n=5), 60 (n=5) and 90 (n=5) days. Control groups were established: stress control (n=5, gastrostomy tube insertion group), absolute control (n=5, untreated group), positive control group (n=5, receives ClB solvent, 0.015% ethanol solution via gastric tube) and negative control group (n=5, receives water via gastric tube). At the endpoints of the experiment (30, 60 and 90 days), after pentobarbital anaesthesia (4.5 mg/b.w. kg, Nembutal, Abbott, USA) the animals were killed and decapitated.

In vitro protocol

Rat HC were separated under a dissecting microscope. From liver tissue by enzymatical (trypsin: 0.2 % /Sigma Aldrich, USA, St. Louis/ for 30 minutes; collagenase / Sigma Aldrich, USA, St. Louis /: 30 µg/mL for 40 minutes; dispase / Sigma Aldrich, USA, St. Louis/: 50 µg/mL for 40 minutes, phosphate buffer was used /PBS-A/; temperature: 37°C) and mechanical dissociation (using a nylon-blutex filter with pore diameter of 48 µm), primary monolayer cell cultures were prepared. Monolayer HC cultures were maintained in Williams' Medium E medium (Sigma, Germany) [10]. Cell proliferation induction was performed on separate reference systems treated with 1 mg/mL benz[c]-acridine (BcA) for 168 h. *In vivo* HC pretreated with ClBs were treated with exposure of ClBs (0.1; 1.0; 10.0 µg/mL) for 168 h.

Mg^{2+}-ATPase activity determination

The Mg^{2+}-dependent ATPase activity after ClB treatment was measured using the modified method of Martin and Dotty to follow discrete energy changes in cell energy transfer. ATPase activity is expressed as control %.

Statistical analysis

To compare the means of different treatment doses (0.1, 1.0, 10.0 µg/b.w. kg) to the controls during 30-, 60-, and 90-days long treatments (n=5 in each group of time and dose) two-way ANOVA was performed. Dose and time were used as the two factors for analysis.
The two-way ANOVA showed a significant difference between the group averages overall ($p<0.001$). Statistical analyses were carried out using IBM SPSS Statistics, version 21 software (IBM Corporation, Armonk, NY, USA).

Results and discussion

According to environmental aspects, if a process originating from an impact factor causes a change in the studied environmental factor or its state, in this case alterations in enzyme activity may be as direct effects. The results are presented to the absolute control group.

Number of tumour clones as a result of ClB treatments

	Hepatocyte
AC	0
BcA	65.7±5.1*

Table 1. The number of tumour clones in response to the absolute reference control (AC) and benz[c]-acridine (BcA) treatment (number of cells±sem, *:p<0.001)

From the data in Table 1, BcA significantly induced tumour clones in the model system, confirming that the experimental setup is suitable for the numerical detection of tumour cells.

	Hepatocyte		
ClB treatment	**30 days**	**60 days**	**90 days**
ClB 10.0 µg/bw.kg	0	1.6± 0.05*	1.9±0.008*
ClB 1.0 µg/bw. kg	0	0.71±0.05*	0.03±1.39*
ClB 0.1 µg/bw.kg	0	0	0.54±0.01*

Table 2. The number of tumour clones in the different cell populations as a result of each ClB treatment (number of cells±sem, *:p<0.001)

The data in Table 2 clearly show that the number of tumour clones in the HC cell cultures increased depending on the dose and time.

Changes in Mg^{2+}-dependent ATPase due to exposure to ClB

The activity of Mg^{2+}-dependent ATPase capable of AC group activity (100%) was significantly increased by benz-(c)-acridine treatment in HC (178.7±8.19%).

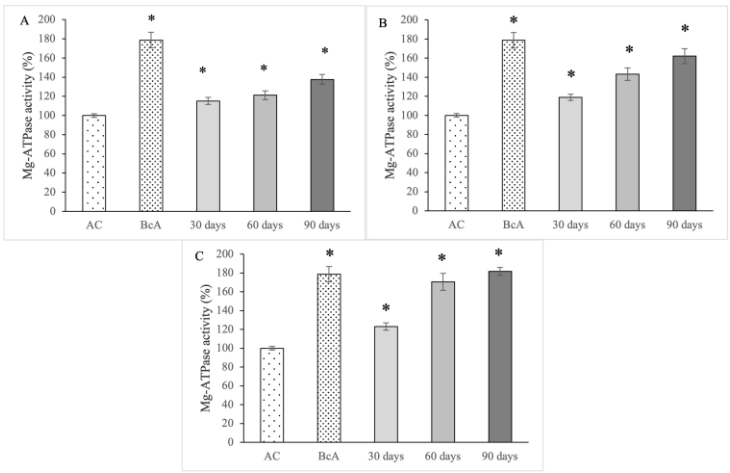

Figure 1. Effects of different exposures to the ClB mix on the activity of Mg^{2+}-dependent ATPase in HC cell cultures (A: 0.1 µg/bw. kg, B: 1.0 µg/bw. kg, C: 10.0 µg/bw. kg doses, *:p< 0.001, n=5/group)

The data in Figure 1 clearly show that the enzyme activity increased significantly (p<0.001) as a function of time and dose. After 90th day of the experiment, 1.0 µg/bw. kg dose exposures (162.1±7.8%) gave a value that approximates the effect of benz-(c)-acridine.
Enzyme activity exceeded (181.7±4.2%) the value of the reference system after 90 day by the effects of a dose of 10.0 µg/bw. kg of ClB.

ClB can also be classified as artificial compounds created during chemical reactions, which can generate changes in many physiological processes. Living systems are affected by complex environmental exposures *in vivo*; however, if these exposures are very low (subtoxic concentrations), they can exert their effects in a latent manner for a long time. If the artificial ClB can generate changes in the central axis and in the peripheral elements, then in a more appropriate approach, we should call these agents homeostatic disruptor compounds. ClB-mix exposures caused a dose-dependent increase in cell proliferation, accompanied by an increase in Mg^{2+}-dependent ATPase activity. However, extremely low doses of ClB treatments did not result in significant changes in transformation activity. According to our morphological observations, ClB treatments induced tumour clone formation in the first generations of primary cell cultures in a time- and dose-dependent manner. As a result of treatment with a higher dose of ClB, tumour clone formation was generated in all primary cell cultures we examined after only 60 days. The initiation effect of ClB was expressed less in the central regulatory elements but more intensively in the peripheral cells. HC cell cultures responded with a sensitive reaction to the chemical agents included in the experiment. If we examine Mg^{2+}-dependent ATPase activity, which indicates the discrete energy transfer of cells, after 30 days all cell types showed an increase in the typical activity because of ClB treatment. The Mg^{2+}-ATPase, located in cell membrane, provides energy through increased hydrolysis of ATP. The release of the energy inherent in the macroergic bond also led to protein production at the cellular level.

Conclusion

The Mg^{2+}-dependent ATPase enzyme shows that the energy from ATP splitting is connected to the cell's own energy supply, and this also manifests itself in increased cell activity. Tumor cells can be characterized by increased cell proliferation activity and increased protein production [10]. This means that ClB included in the model study have an effect on both initiation and promotion. Thus, it can be established that ClBs exert a general effect on cells.

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