University of Szeged
Faculty of Science and Informatics
Doctoral School of Environmental Science
Department of Environmental Biology and Education

ENVIRONMENTAL CONDITION ALTERATIONS AND BIOLOGICAL SYSTEM RESPONSES

Abridgement of the Ph.D Thesis

Molnár Zsolt

Instructors:

Dr. habil Gálfi Márta Ph.D.
College Professor and Head of Department

Dr. Valkusz Zsuzsanna Ph.D.
Clinical Head Physician

Szeged

2015

INTRODUCTION AND AIMS

Biological objects exist in determined environmental condition. These objects take inputs from their environment to maintain their inner homeostatic balance, which can induce the system accommodation processes. Persistent interaction evolves among living creatures and their environment. This connection among the biotic and abiotic systems can materialize e.g. with the neuro-endocrine communication. Nowadays the most complex evolutionary scene is the society, in which the human performers to maintain their existence can modify their environment.

The endocrine disruptor compounds (EDC) are xenobiotics produced by the chemization. However the society to supply its wants and needs can alter its environment. These condition alterations can modify the accommodation patterns of living organisms, e.g. EDC can alter the neuro-endocrine balances and extracellular ion milieu. Various benzene derivates such as alkylbenzenes and chlorobenzenes (ClB), however, continue to be used as chemical intermediates, solvents, pesticides in spite of incomplete knowledge of their chronic toxicity. Several chlorinated benzenes are known to be porphyrogenic, carcinogenic, mutagenic in animals and humans. In this context the EDC are homeostatic disruptor (HD) agents.

Steroid hormones, mainly estrogen, are known to modify the neuro-endocrine system. A rapid increase in the rate of prolactin (PRL) synthesis was detected both in vivo and in vitro with estradiol treatment. Recent papers have revealed that estrogen may induce prolactinoma (PRLoma) by stimulating cell signaling mechanisms such as protein kinase activity, thus potentiating hormone exocytosis.

The cell and its external milieu comprise an operational unit as a persistent and dynamic contact evolves among the living structure and its environment. The extracellular ion milieu determines the endo- and exocytosis mechanisms.

Our aims were:

1. We wanted to investigate the cellular mechanisms under hypokalaemic effects, because the effect of extracellular hypoionic conditions on cellular functions is intriguing and likely important factor in a number of pathologies.

    a. To investigate cellular phenomena, we wanted to develop *in vitro* model systems, namely primary monolayer adenohypophysis (AdH) and PRLoma cell cultures and their cellular function were wanted to standardize.
    b. We intended to investigate response mechanisms in the function of normal, monolayer, primary AdH cell cultures at low extracellular [K^+].
    c. We also wanted to investigate the hormone release of PRLoma at different extracellular K^+ milieu, ranging from low to normal K^+ levels.
    d. We also examined the mechanisms for the extracellular hypokalaemic effects on hormone exocytosis by the altered regulated, transformed cell populations.

2. The environmental pollution by HD agents may have chronic and/or acute effects on the living organisms.
    a. We aimed to develop a standardized *in vivo* and *in vitro* research model to study the subtoxic concentration and the expositor role of chlorobenzenes (ClB).
    b. This paper focus on the central adrenocorticotropic hormone (ACTH) release in the pituitary by the effects of combined, extremely low-dose, long-term, pre-standardized ClB treatment.
    c. We wanted to draw attention the effects of subtoxic exposition of ClB on the Mg^{2+}-dependent ATPase activity.
    d. We endeavoured to examine the morphology and structure of AdH tissues by the effects of combined exposure of ClB treatment.

MATERIAL AND METHODS

Female and 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 in the experiment. The animal care and research protocols were in full accordance with the guidelines of 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. Experimental animals lived under automated diurnal conditions (12 h dark and 12 h light system) in groups of 10 animals for 6 months. Standard pellet food and tap water were available *ad libitum.*

Prolactinoma induction and in vitro experiments

Female Wistar rats (n=20) were treated subcutaneously with estrone-acetate (CAS registry number: 901-93-9, Sigma, Germany; 150 μg/kg b.w./week) for 6 months to induce adenohypophyseal prolactinomas.

After pentobarbital anaesthesia (4.5 mg/kg b.w. Nembutal, Abbott, USA) the animals were killed and decapitated. Tissues were separated under a preparative microscope. Primary, monolayer cell cultures were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2 % /Sigma, Germany/ for 30 min; collagenase /Sigma, Germany/: 30 μg/ml for 40 min; dispase /Sigma, Germany/: 50 μg/ml for 40 min in phosphate-buffered saline /PBS-A/; temperature: 37°C). Mechanical dispersion was achieved with nylon blutex sieves (Ø: 83 and 48 μm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be 2x105/cm3. The dissociated cells were placed onto 24 well-plastic plates (5% collagen coated /Nunc., Geramany/; Dulbecco's Modified Essential Medium /DMEM/ + 20% Fetal Calf Serum /FCS/ + antibiotics /Penicillin+Streptomycin: 1.0 μg/ml/). The cells were cultured at 37 °C in a CO2 incubator that provided a humidified environment of 95% air and 5% CO2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking for PRL and ACTH protein release . After functional standardization, the basal ACTH and PRL levels were determined in both normal Adh and PRLoma (Tyrode's medium /Sigma, Germany/). In the medium, only the [K+] was modified; all other essential anions and cations were under homeostatic (e.g. isoionic) conditions. The hormone release of primary cell cultures was detected under hypokalaemic conditions of varying degrees ([K+]: 0; 0.5; 1.0; 1.5; 2.0 mM; n=10 in each group). Samples were taken at 10, 20, 30, 60 and 90 minutes after treatments to measure hormone kinetics.

The PRL and the ACTH content were detected in the supernatant media. From the supernatant media, 500 µl samples were removed at appropriate times and stored at -80°C. The prolactin (PRL) was determined with radioimmunoassay (RIA) and the adrenocorticotropic hormone (ACTH) was measured with immuno-chemiluminescence assay (LIA).

A modified Lowry Method [26] and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

Immunocytochemistry

Immunostaining with anti Bcl-2 (Santa Cruz Biotechnology Inc., 1:25 dilution, N-19, sc-492) for 60 min was performed (samples were washed in Tris buffered saline /TBS, 0.05 M, pH 7.4 and 0.85% NaCl/ for 5 min before treatment) after incubating of monolayers with peroxidase blocking reagent for 5 min. After additional washes TBS (5 min) bound antibodies were visualized using 3,3'-diaminobenzidine tetra-hydrochloride (DAB, Sigma, Germany) for 2 min.

Statistical analysis

To compare various effects of treatment in Adh and PRLoma over time, two-way repeated measures ANOVA was used. Significant interaction was found between the two investigated factors ($p<0.001$), thus both effects could not be reported independently.

Experimental protocol for the examination of homeostatic disruptor agents

In vivo research protocol

Male Wistar rats were treated with combined ClB (1:1 mixture of 1,2,4- trichlorobenzene and hexachlorobenzene 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. The rats were exposed to ClB for 30 (n=10), 60 (n=10) and 90 (n=10) days. Control groups were set up: stress control (n=5, gastrostomy tube insertion group) and absolute control (n=5, untreated group). At the endpoints of the experiment (30, 60 and 90 days), blood samples were taken and serum was separated and stored at −70℃until measurements.

In vitro experimental protocol

After pentobarbital anaesthesia (4.5 mg/b.w. kg, Nembutal, Abbott, USA), at the endpoints of the research (30, 60, and 90 days) the animals were killed and decapitated. Primary, monolayer adenohypophysis cell cultures (AdH) were prepared by enzymatic and mechanical dissociation as described above.

Determination of ACTH and Mg^{2+}-ATPase activity measurement

The ACTH levels of blood serum and supernatant media were measured by LIA with an Immulite 2000 apparatus (Siemens Healthcare Diagnostic, Deerfield, IL) and DPC kit (L2KAC-02; Euro/DPC Ltd, Glyn Rhonwy, UK).

A modified Lowry Method and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA) were used for the determination of total protein content.

The Mg^{2+}-dependent ATPase activity after ClB treatment was measured by the modified method of Martin and Dotty.

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=10 in each group of time and dose) two-way ANOVA were run. For all three variables (ACTH levels of cell cultures, ACTH levels of serum and Mg^{2+}-ATPase activity) two-way ANOVA resulted a significant p-value ($p<0.001$), reflecting, there is an overall difference between the group means.

DISCUSSION IN THE LIGHT OF RESULTS

Differences between the effects of hypokalaemia on normal adenohypophysis cultures and prolactinoma cell populations were investigated. Significant alteration ($p<0.001$, $n=10$) in hormone exocytosis was detected in K^+ treated adenohypophyseal and prolactinoma cell cultures compared to untreated groups. According to our results under higher, but still hypokalaemic conditions the PRL release was reduced depending on duration of exposure in AdH. The PRL release was reduced in PRLoma cell populations compared with the control groups. In these processes the accommodation was played a key role. The decrease in hormone exocytosis was tightly correlated to the extracellular K^+ in both cell types, leading to the conclusion that external K^+ may be the major factor for the inhibition of hormone release.

We next examined the kinetics of ACTH release under different [K^+]. The hormone concentration in the supernatant media was reduced significantly in AdH compared with control groups. The ACTH release in PRLoma was also presented under different [K^+] exposition. The ACTH release of PRLoma was increased significantly depending upon the duration of experiment. In the 1.5 and 2.0 mM [K^+] manipulated groups the ACTH secretion was decreased depending upon the duration of exposure. The hormone levels in the PRLoma group treated with 1.0, 0.5 and 0 mM [K^+] were elevated significantly compared to control groups.

We examined the correlation between the normal and the altered endocrine regulation modified by hypokalaemia. Under hypokalaemia the ACTH release of PRLoma was increased significantly, in contrast to control AdH. A similar interaction is depicted in the PRL release because the PRL content of supernatant media increased significantly ($p<0.001$) in PRLoma compared with control AdH. According to our hypothesis the cell aging machinery may play role in this phenomenon.

The Bcl-2 production was investigated and immunocytochemistry showed that Bcl-2 expression was reduced under hypokalaemic conditions.

We also wanted to investigate the discrete balance alteration by the effects of HD agents. The applied doses of ClB (0.1, 1.0 and 10.0 μg/ b.w. kg) modified the ACTH release of serum and the monolayer cell cultures depending on the duration of exposure. The subtoxic exposure of ClB enhanced significantly the ACTH levels compared with the control groups. Notable enhancement was observed in the Mg^{2+}-dependent ATPase enzyme activity compared with the control. According to immunohistochemistry, the pituitary tissue in control groups

revealed a normal histological structure. In contrast, in the ClB treated groups intense immunoreaction was observed for ACTH.

Based on the findings, we grouped our results and conclusions around the topics defined in the objectives of this research. Our conclusions are summarized:

1. Our investigations on the cellular alterations caused by hypokaleamia are given new information.
    a. The cell and its environment can be characterized by determinative system roles. Changes in the system structure are mediated by the control and the regulation. The regulation exerts to conserve the system in its current stage while the control leads it through the stages of development. The control in the alteration of extracellular ion milieu forces immediate response mechanisms that can be detected in the level of regulation by the modification of hormone release. Since the extracellular environment is under persistent fluctuation, thus, the cell has to adapt to it (hyperpolarization and apoptosis). To investigate these mechanisms *in vitro* and *in vivo* research protocols were made.
    b. The extracellular ion milieu ensures the sign and maintaining inputs, which realize the accommodation of normal and transformed cell populations.
    c. According to the literature around the environment of different tumour hypoionic conditions can be detected related to essential monovalent cations. In our research protocol it is postulated that the induced apoptosis by hypokaleamia deputizes the system's defensive function.
    d. Our preliminary studies may give possibilities to design therapeutic protocols to treat diseases and cancers that that affect endocrine tissues.

2. The ClB are HD agents however, continue to be used as chemical intermediates, solvents, and pesticides in spite of incomplete knowledge of their chronic toxicity, thus *in vivo* and *in vitro* model systems were developed and standardized for the investigation of these agents.
    a. The extreme low, subtoxic dose of ClB, as chemical stressors modified the ACTH release *in vivo* and *in vitro* via the simulation of the most common exposition pathway (food chain).
    b. Our results showed that the applied doses of ClB have direct and indirect affect on the biological systems.

c. The HD agents altered the discrete energy transfer via the modification of Mg^{2+}-dependent ATPase activity.
d. The chronic and subtoxic doses of ClB treatments structural alterations were detected in the histology of AdH, which were caused by the proliferative manner of these persistent organic compounds.

LIST OF PUBLICATIONS RELEVANT TO THE SUBJECT

[1] Molnar Z, Palfoldi R, Laszlo A, Radacs M, Sepp K, Hausinger P, Tiszlavicz L, Valkusz Z, Galfi M. The effects of chronic and subtoxic chlorobenzenes on ACTH release. JOURNAL OF ENVIRONMENTAL SCIENCES (2015)

Impact factor: 1,922 (Közlésre elfogadva)

[2] Molnar Z, Palfoldi R, Laszlo A, Radacs M, Laszlo M, Hausinger P, Tiszlavicz L, Razga Z, Valkusz Z, Galfi M. The Effects of Hypokalaemia on the Hormone Exocytosis in Adenohypophysis and Prolactinoma Cell Culture Model Systems. EXPERIMENTAL AND CLINICAL ENDOCRINOLOGY & DIABETES 122: p. 575. (2014)

Impact factor: 1,760

[3] Kis GK, Ocsko T, Galfi M, Radacs M, Molnar Z, Rakosi K, Molnar AH, Laszlo F, Varga C, Laszlo FA: The effects of orexins on monoaminerg-induced changes in vasopressin level in rat neurohypophyseal cell cultures. NEUROPEPTIDES 45:(6) pp. 385-389. (2011)

Impact factor: 2,129