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Effects of chronic and subtoxic chlorobenzenes on adrenocorticotrophic hormone release

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ABSTRACT

Many environmental chemicals and pesticides have been found to alter neuroendocrine communication in exposed biological objects. The environmental loads have primary and secondary effects that can alter the homeostatic regulation potential. Since it is difficult to avoid human exposition, a potentially important area of research to develop *in vivo* and *in vitro* experimental models. In this context, the primary aim of this study was to demonstrate the effects of chlorobenzenes on adrenocorticotrophic hormone (ACTH) release. In our experimental study, male Wistar rats were exposed to 0.1, 1.0 and 10 μg/b.w. (body weight) kg of 1,2,4-trichlorobenzene and hexachlorobenzene (ClB) mix *via* gastric tube for 30, 60 or 90 days. At the endpoints of the experiment blood samples were taken and animals were decapitated. Primary, monolayer adenohypophysis cell cultures were prepared by enzymatic and mechanical digestion. The ACTH hormone content in serum and supernatant media was measured by immuno-chemiluminescence assay. The Mg^{2+}-dependent ATPase activity was determined by modified method of Martin and Dotty. Significant differences were detected in the hormone release between the control and treated groups. The hormone release was enhanced characteristically in exposed groups depending upon the dose and duration of exposure. The Mg^{2+}-ATPase activity enhanced after chronic and subtoxic ClB exposition. Light microscopy revealed that the adenohypophysis seemed to be more abundant. Results indicate that Wistar rats exposed to subtoxic ClB have direct and indirect effects on hypothalamus–hypophysis–adrenal axis.

Introduction

Due to rapid industrialization, acute and chronic poisoning with pesticides is a global public health problem. One group of pesticides is the persistent organic pollutants (POP), which can interfere with the endocrine communication that called endocrine disruptor compounds (EDC). These chemicals are xenobiotics that are proven to have effects

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on homeostasis and/or its regulation. The toxicity of POP depends on the effects of their hazardous nature, in combination with high chemical and biological stability, and a high degree of lipophilicity (Colborn et al., 1993). Various benzene derivates such as alkylbenzenes and chlorobenzenes (ClBs), however, continue to be used as chemical intermediates, solvents, pesticides in spite of incomplete knowledge of their chronic toxicity. Several of the chlorinated benzenes are known to be porphyrogenic, carcinogenic, mutagenic and can interfere with the endocrine system in exposed animals and humans (Bigsby et al., 1999; Oehlmann et al., 2000).

Most papers showed only the contact and toxic effects of ClB, although these agents accumulate in adipose tissues and cause indirect alterations in exposed biological objects (De Bleecker et al., 1992; Raymond-Delpech et al., 2005). ClB as stressors may activate the hypothalamic–pituitary–adrenal (HPA) axis in exposed mammals, which play a role in the adaptation processes. Bousquet et al. (2001) showed that central and peripherally derived stressors activate neuroendocrine cascade mechanisms in the paraventricular nucleus. The corticotropin-releasing hormone (CRH) activates the corticotroph cells in the anterior lobe of the pituitary gland, which stimulate the expression of proopiomelanocortin (POMC), the precursor of adrenocorticotropic hormone (ACTH) (Karalis et al., 2004; Liu et al., 2003). On the other hand there is grooving evidence that arginine-vasopressin (AVP) is involved in the physiologic regulation of ACTH *via* Ca^{2+} dependent receptor V1b (Gibbs et al., 1986; Legros, 2001; Lolait et al., 1995). The expressed ACTH is crucial for the development of adrenal cortex and plays an essential part in the regulation of androgens and glucocorticoids (Beuschlein et al., 2001).

Reinhart (1988) showed that Mg^{2+} has both a direct effect on Ca^{2+}-channels and an indirect effect by stimulation of protein phosphatases that dephosphorylate Ca^{2+}-channels leading to enhancement of Ca^{2+} influx; thus Mg^{2+}-dependent ATPase plays a role in discrete energy transfer and in triggering exocytosis.

This article focuses on the central ACTH release in the pituitary by the effects of subtoxic exposition of ClB. We wanted to draw an attention to the effects of subtoxic exposition of ClB on ACTH release and Mg^{2+}-dependent ATPase activity. For this reason, we aimed to develop a standardized *in vivo* and *in vitro* research model to study the basic regulation of ACTH. The aim of this study was also to investigate the structural and biochemical alterations of the anterior pituitary of male Wistar rats after subtoxic exposure to ClB.

1. Materials and methods

1.1. Animals

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 hr dark and 12 hr light system) in groups of 10 animals. Standard pellet food and tap water were available *ad libitum*.

1.2. 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°C until measurements. After pentobarbital anesthesia (4.5 mg/b.w. kg, Nembutal, Abbott, USA) the animals were killed and decapitated.

1.3. *In vitro* experimental model

Adenohypophysis and neurohypophysis were separated under a preparative microscope. Primary, monolayer adenohypophysis cell cultures (AdH) were prepared by enzymatic and mechanical dissociation. The tissues were digested enzymatically (trypsin: 0.2%/Sigma, Hamburg, Germany/for 30 min; collagenase/Sigma, Hamburg, Germany/: 30 μg/mL for 40 min; dispase/Sigma, Hamburg, Germany/: 50 μg/mL for 40 min in phosphate-buffered saline/PBS-A/; temperature: 37°C). Mechanical dispersion was achieved with nylon blutex sieves (∅: 83 and 48 μm). Cultures were controlled for both viability (>95%; trypan blue exclusion) and function and the cell density was determined to be 2×10^5/mL. The dissociated cells were placed onto 24 well-plastic plates (5% collagen coated/Nunc., Orlando, FL, USA/; Dulbecco's Modified Essential Medium/DMEM/(Sigma, Hamburg, Germany) + 20% Fetal Calf Serum/FCS/(Gibco, New York, NY, USA) + antibiotics/penicillin + streptomycin: 1.0 μg/mL). The cells were cultured at 37°C in a CO_2 incubator that provided a humidified environment of 95% air and 5% CO_2. The medium was changed every 3 days. Primary cell cultures were standardized by immunohistochemical methods, marking ACTH protein release. After functional standardization, the basal ACTH level was determined in Adh.

1.4. Adenohypophysis immunohistochemistry

The adenohypophysis was removed and separated from neurohypophysis, immediately dehydrated through an ascending ethanol series, fixed in 4% formalin solution and embedded in paraffin. After embedding in paraffin, tissue sections were deparaffinized 2 times with xylene (Sigma, Hamburg, Germany) for 10 min and 2 times with ethanol (Sigma, Hamburg, Germany) for 5 min. The deparaffinized sections were contour stained with hematoxylin and eosin. The indirect immunohistochemical method was carried out by incubating the sections overnight at room temperature using rabbit ACTH polyclonal antibody (dilution 1:500, Phoenix Pharmaceuticals, Inc.). Peroxidase activity was revealed with