

Research Article

The Role of Uron and Chlorobenzene Derivatives, as Potential Endocrine Disrupting Compounds, in the Secretion of ACTH and PRL

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Uron herbicides polluting the environment represent a serious concern for environmental health and may be regarded as endocrine-disrupting compounds (EDCs), which influence the regulation of human homeostasis. We aimed to investigate the effect of EDC urons (phenuron: PU, monuron: MU, and diuron: DU) and chlorobenzenes on the basal release of the adrenocorticotrophic hormone (ACTH), which is a part of the adenohipophysis-adrenocortical axis. Hormone secretion in the presence of EDC was studied in two cell types: normal adenohipophysis cells (AdH) and cells of prolactinomas (PRLOMA). PRLOMA was induced in female Wistar rats by subcutaneously injecting them with estrone acetate for 6 months. AdH and PRLOMA were separated from treated and untreated experimental animals, dissociated enzymatically and mechanically in order to create monolayer cell cultures, which served as an experimental *in vitro* model. We investigated the effects of ED agents separately and in combination on ACTH and prolactin (PRL) release through the hypophyseal-adrenal axis. Hormone determination was carried out by the luminescent immunoassay and the radioimmunoassay methods. Our results showed that (1) uron agents separately did not change ACTH and PRL release in AdH culture; (2) ACTH secretion in arginine vasopressin-(AVP-) activated AdH cells was significantly increased by EDC treatment; (3) ED agents increased the basal hormone release (ACTH, PRL) in PRLOMA cells; and (4) EDC exposure increased ACTH release in AVP-activated PRLOMA cells. We conclude that the herbicides PU, MU, and DU carry EDC effects and show human toxicity potential.

1. Introduction

Chemical agents (e.g., halogenated hydrocarbons and uron herbicides [1–3]) which pollute the environment represent a serious concern for environmental health [4, 5] and may be regarded as endocrine-disrupting compounds (EDCs), which influence the regulation of human homeostasis [6]. They may change the potential [7, 8] and capacity [9, 10] of the psycho-neuro-endocrine-immune regulation network and may bring about disturbances in the regulatory

process [11, 12] which result in serious homeostatic alteration even in healthy individuals [13, 14]. Human adaptation patterns induced by environmental burdens are obviously modified when the exposition reaches an individual who suffers from functional and/or structural disorders (illnesses) [15, 16]. An open dynamic system which forms a unit with its environment, for example, a living human organism, can stabilize its equilibrium processes as determined by the direct environmental conditions (attraction range) [17] defined by its genetic and functional

adaptation potential (algorithmic networks characterizing the local properties of the living system [18]). In human homeostasis, hormones are the creative elements of the neuroendocrine regulation [19, 20]. Human neuroendocrine regulation can be interpreted as a network of open, dynamic biological systems [21] in the outlined context. Biological cycles that can be described with the “AND” function are those essential for life (e.g., human hypothalamus-adenohypophysis-adrenal cortex axis functional disorder) [22]. The “OR” function-related control systems are not essential at the organizational level of the given individual (e.g., PRL); life functions can be maintained in their absence. The disturbance of the healthy (control) processes of the “OR” cycle will affect the “AND” cycles. Chronic changing of the “OR” cycle may lead to structural disturbance, for example, cellular proliferation, which is sustained by continuous feedback information [23].

Herbicides [24] and halogenated hydrocarbons [25] are widespread substances that have an EDC effect. Phenuron (PU), monuron (MU), and diuron (DU) compounds may be viewed as a halogenated homologous series of herbicides [26]. Chlorobenzenes (ClB) represent a halogenated aromatic hydrocarbon group, of which 1,4-dichlorobenzene (dClB) is known as an international reference compound due to its ecotoxicological and human toxicity potential [27]. It is a primary question from the point of view of medical practice, whether the cellular follow-up of ACTH regulation is a suitable test system for studying the effect of the environmental pollutants. Any exposure can be interpreted as a stressor in the human neuroendocrinium [28]. The outlined circuit (in mathematical terms: attractor) maintains processes indispensable in human homeostasis, since there is no life without adrenocortical hormones. The regulatory disturbances (e.g., feedback disturbances) may become causative factors, for example, they may lead to benign cell proliferation. The most commonly occurring human adenohypophysis cell proliferation is prolactinoma, which generates prolactin overproduction. In the development of the tumor, a significant pathophysiological role is attributed to the estrogenic effect [29].

When investigating the neuroendocrine aspects of ED agents (e.g., uron herbicides and ClB), the experimental layout is based on preliminary experiments (dose and time kinetics) [30] and the already proven physical, chemical, and biological effects of the compounds. Urons are substituted phenylureas of high chemical stability, which are used in agriculture as photosynthesis-inhibiting herbicides [31]. Due to their long chemical half-life (a few months to one year in soil, 2–6 weeks in water), the food chain can be severely affected [32, 33]. In humans, low to moderate toxicity is associated with spleen and liver involvement, whereas carcinogenicity [34] has also been demonstrated, and in the case of diurons, antiandrogenic properties have been described as well [35, 36]. Chlorobenzenes are also highly persistent chlorine-substituted aromatic hydrocarbons forming a homologous series [2, 37, 38]. Toxicity studies report liver and kidney impairment, but their roles as carcinogenic (breast, liver, and kidney) agents are also known [39, 40]. As EDC effect, the alterations of thyroid hormone synthesis

and androgenic functions have been discovered [41, 42]. The human toxicity potential (HTP) is an internationally standardized impact category set in LCA (life cycle analysis) standards, used to express various environmental effects [43], in which the effects of environmental 1,4-dichlorobenzene on human health are the benchmark. The determination and relation of ecotoxicity potential (ETP) to dClB effects are performed in a similar fashion.

Our aims were to investigate the effect of ED compounds ((PU, MU, and DU) and dClB and 1,2,4-trichlorobenzene + hexachlorobenzene (chlorobenzene mixture, mClB)) on the basal release of the ACTH involved in the functioning of the adenohypophyseal-adrenocortical axis. This work focused on hormone secretion in the presence of EDC in two cell types: normal adenohypophysis (AdH) cells and prolactinoma (PRLOMA) cells. In this regard, the question was, Can EDC modify the feedback mechanism of ACTH release governed by arginine vasopressin (AVP) and corticosterone (B) (“AND” cycles)? Observing EDC effects on PRL hormone secretion was also a goal in this research (“OR” cycle).

2. Materials and Methods

2.1. Experimental Animals. Certified healthy female rats were used in our experiments (Wistar strains weighing 120–250 g, 4–6 weeks old at the onset of the study) (Charles River, Isaszeg, Hungary). During the experimental period, animals were kept in a controlled (55–65% relative humidity, $22 \pm 2^\circ\text{C}$ air temperature), automated diurnal environment (12 h daytime, 12 h night illumination cycles) in $32 \times 40 \times 18$ cm cages (5 animals/cage). The diet required for experimental animals (CRLT/N, Charles River, Hungary) and drinking water were available ad libitum. The animals involved in the study were treated in accordance with Gov. Ordinance Number 40/2013 (II. 14.) on animal experiments.

2.2. Induced Prolactinoma and In Vitro Experimental Models. The PRLOMA models were made from Wistar rats (♀ , $n = 20$) which were subcutaneously injected with estrone acetate for 6 months (CAS registration number 901-93-9, Sigma, Germany, $150 \mu\text{g}/\text{kg}/\text{week}$) [44]. After the pentobarbital ($4.5 \text{ mg}/\text{kg}$, Nembutal, Abbott, USA) anesthesia, the animals were decapitated and AdH was separated; the tissue was enzymatically (trypsin: Sigma, Germany, 0.2% for 30 min; collagenase: Sigma, Germany, $30 \mu\text{g}/\text{ml}$ for 40 min; dispase: Sigma, Germany, $50 \mu\text{g}/\text{ml}$ for 40 min; phosphate-buffered saline (PBS-A) was used for the solutions, temperature: 37°C) and mechanically ($83 \mu\text{m}$ and $48 \mu\text{m}$ pore size nylon blutex filter) dissociated. The cell viability was $\geq 95\%$ (trypan blue staining). The cells were suspended in the following medium: Dulbecco's Modified Essential Medium (DMEM, Sigma, Germany) + 20% fetal calf serum (FCS, Sigma, Germany) + 1.0 IU/ml penicillin + streptomycin (Sigma, Germany). Then, they were placed into surface-treated (5% collagen) 24-well plastic culture vessels (Nunc, Germany) and put in a thermostat (temperature = 37°C , pCO_2 : 5%). Cell cultures were washed every 3 days after adherence until they became confluent.

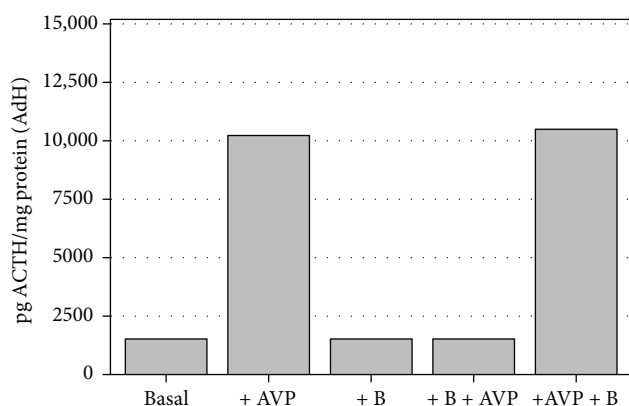


FIGURE 1: Functional assay for ACTH release in normal rat adenohypophysis cultures, *in vitro* ($n = 8 - 12$ in each group); mean level \pm SEM (pg ACTH/mg protein): AVP significantly increases ($p < 0.001$), whereas corticosterone alone does not alter ACTH release when compared to control; preincubation with +B inhibited AVP activation (basal: control; AVP: 8-arginine vasopressin; B: corticosterone).

Specific functional standardization for ACTH release was regulated by $1 \mu\text{g/ml}$ corticosterone (B) and 10^{-6} M AVP treatments. The ACTH release cycle (“AND” cycles) was activated by AVP; this mechanism was inhibited by a 20-minute preincubation with B (Figures 1 and 2).

2.3. Experimental Protocol. Time and dose kinetic assays, determining the appropriate arrangements, were performed on standardized AdH and PRLOMA *in vitro* cultures. In the present study, AdH and PRLOMA cell cultures were first treated for 60 minutes with chlorobenzene (dClB = 0.1 ng/ml ; chlorobenzene mix (mClB) = 0.1 ng/ml ; hexachlorobenzene and 1,2,4-trichlorobenzene 1:1) and with urons (PU: 10^{-6} M, MU: 10^{-6} M, DU: 10^{-6} M). At the end of the EDC treatment, samples were obtained from the supernatant media of the cell cultures for the determination of ACTH and PRL hormones. When studying the ACTH-mediated role of EDC agents in the hypophysis/adrenal cortex regulation, the EDC agents were coadministered with (10^{-6} M) AVP and after the 60-minute treatment period, samples were taken from the supernatant media of both AdH and PRLOMA, in the regulation cycle of AVP/B feedback studies in Figures 1 and 2.

EDCs were added together with B. AVP was administered after a 20-minute pretreatment with EDC + B, and at the end of the treatment period, the supernatant media were sampled. In order to follow PRL hormone release, AdH and PRLOMA cell cultures were individually treated with ED agents for 60 minutes. Next, the supernatant media were used to measure PRL.

2.4. Hormone Assays. PRL assay was performed by radioimmunoassay from samples obtained according to the experimental protocol [44]. Determination of ACTH from samples was carried out by the luminescent immunoassay method, using the apparatus of the Endocrinology Unit, First Dept. of Internal Medicine, Faculty of Medicine, University

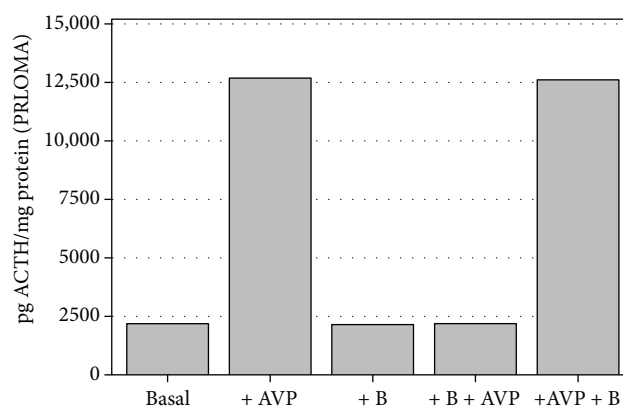


FIGURE 2: Functional assay for ACTH release in rat prolactinoma cultures (PRLOMA), *in vitro* ($n = 11 - 12$ in each group); mean level \pm SEM (pg ACTH/mg protein): compared with normal AdH cells (Figure 1). ACTH release always shows a similarly significant ($p < 0.001$) increase in PRLOMA cells (basal: control; AVP: 8-arginine vasopressin; B: corticosterone).

of Szeged (Immulate 2000, Siemens Healthcare Diagnostic, Deerfield, IL, USA and DPC kit/L2KAC-02, Euro DPC Ltd., Glyn Rhonwy, United Kingdom). The protein content of the samples was determined using a modified Lowry method [45] and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, USA).

2.5. Statistical Analysis. Measurements ($n = 8 - 12$ per group on 24 lots: pooled samples on AdH cell cultures) of ACTH and PRL hormone release by disease (PRLOMA versus normal AdH) in various EDC groups (control, dClB, mClB, PU, MU, and DU) by regulation (basal, +AVP, +B, +B +AVP, and +AVP+B) were compared using mixed models on rats [46, 47]. The regulation cycle was verified in a mixed model for the comparison of the control groups of EDC for ACTH in the 5 regulation phases, using disease and regulation as fixed effects and random intercept for the lots. For ACTH data, a mixed model was applied with disease, EDC, and regulation (only basal, +AVP, and +B+AVP) as fixed factors and random intercept for the lots. For PRL measurements, a mixed model was applied with disease and EDC as fixed factors and random intercept for the lots for basal regulation data. In the analysis models, the reference group was the normal (healthy AdH), control (no EDC treatment), and basal (no regulation) group. Restricted maximum likelihood estimation and Kenward-Roger method for adjusting the degrees of freedom were applied in all models with unstructured covariance matrix. Pairwise comparisons were estimated by least squares means using Sidak p value adjustment. Model residuals were displayed on quantile-quantile plots to check normality assumptions. Statistical analyses were performed in SAS (version 9.3 SAS Institute Inc., Cary, NC, USA), where p values of < 0.05 were considered to indicate statistical significance [48].

3. Results

Figure 3 shows the effect of various ED compounds (dClB, mClB, PU, MU, and DU) on ACTH release in AdH cultures

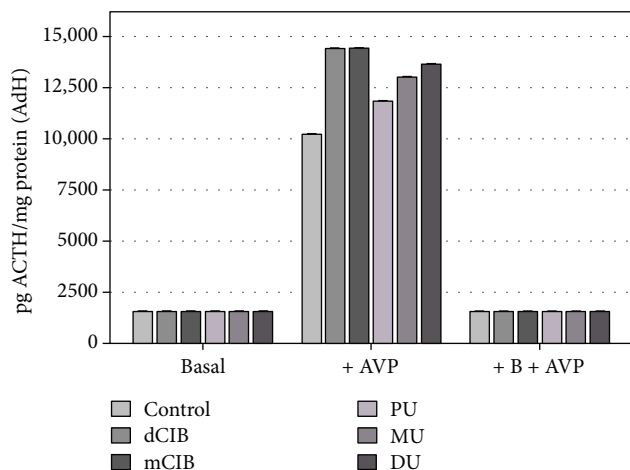


FIGURE 3: EDC effects in the regulation of ACTH release on normal rat AdH cultures, *in vitro*. Mean (ACTH level) \pm SEM. The mean and SEM are calculated from $n=12$. Abbreviations: B = corticosterone: $1 \mu\text{g/ml}$; AVP = 8-arginine vasopressin: 10^{-6} M, +B+AVP: in combination therapy B precedes AVP administration by 20 minutes; dCIB = 1,4-dichlorobenzene: 0.1 ng/ml; mCIB = chlorobenzene mix: 0.1 ng/ml; PU = phenuron: 10^{-6} M; MU = monuron: 10^{-6} M; DU = diuron: 10^{-6} M. All EDC groups differ significantly from the control ($p < 0.001$) for +AVP.

in the following cases: basal, AVP activated (+AVP), and the corticosterone-inhibited AVP activation (+B+AVP) in AdH cultures. It can be seen that ACTH release was not altered by ED agents (mean level \pm SEM (pg ACTH/mg protein): dCIB 1567.91 ± 3.09 ; mCIB 1585.33 ± 2.72 ; PU 1533.67 ± 2.52 ; MU 1553.17 ± 3.40 ; and DU 1566.33 ± 2.30) compared to the control group (1528.25 ± 6.14 pg ACTH/mg protein). In the AVP-activated samples, ACTH release showed a significant increase compared to the control group for each EDC (mean level \pm SEM (pg ACTH/mg protein): control $10,220.88 \pm 20.36$; dCIB $14,430.08 \pm 3.01$; mCIB $14,488.90 \pm 3.57$; PU $11,845.67 \pm 7.02$; MU $13,008.25 \pm 10.18$; and DU $13,658.75 \pm 15.83$). In the case of regulatory effect (+B+AVP-feedback), large deviations could not be detected in the presence of EDC (mean level \pm SEM (pg ACTH/mg protein): control 1524.67 ± 3.46 ; dCIB 1542.00 ± 1.22 ; mCIB 1566.92 ± 2.40 ; PU 1540.08 ± 2.86 ; MU 1560.08 ± 1.88 ; and DU 1578.08 ± 1.26).

Figure 4 shows the effects of dCIB, mCIB, PU, MU, and DU on ACTH release in PRLOMA cultures in the following cases: basal, AVP activation (+AVP), and corticosterone-inhibited AVP activation (+B+AVP). It can be seen that ED agents modulate ACTH release compared to the control of the basal group (2193.64 ± 1.92 pg ACTH/mg protein): dCIB: 2624.30 ± 7.60 pg ACTH/mg protein; mCIB: 2956.08 ± 4.71 pg ACTH/mg protein; PU: 2427.33 ± 6.08 pg ACTH/mg protein; MU: 2535.17 ± 5.14 pg ACTH/mg protein; and DU: 2705.33 ± 4.63 pg ACTH/mg protein. AVP-activated ACTH release of PRLOMA cultures shows a significant increase for each ED compound used (mean level \pm SEM (pg ACTH/mg protein): control $12,674.50 \pm 7.23$; dCIB $14,620.58 \pm 5.61$; mCIB $14,830.50 \pm 7.42$; PU $13,129.67 \pm 6.06$; MU $14,954.17 \pm 11.22$;

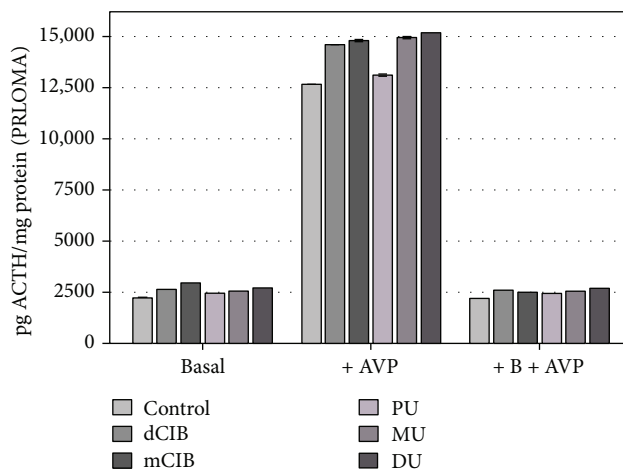


FIGURE 4: EDC effects in the regulation of ACTH release in rat PRLOMA cultures, *in vitro*. Mean (ACTH level) \pm SEM. The mean and SEM are calculated from $n=12$. Abbreviations: B = corticosterone: $1 \mu\text{g/ml}$; AVP = 8-arginine vasopressin: 10^{-6} M, +B+AVP: in combination therapy B precedes AVP by 20 minutes; dCIB = 1,4-dichlorobenzene: 0.1 ng/ml; mCIB = chlorobenzene mix: 0.1 ng/ml; PU = phenuron: 10^{-6} M; MU = monuron: 10^{-6} M; DU = diuron: 10^{-6} M. All EDC groups differ significantly from the control ($p < 0.001$) for basal, +AVP, and +B+AVP.

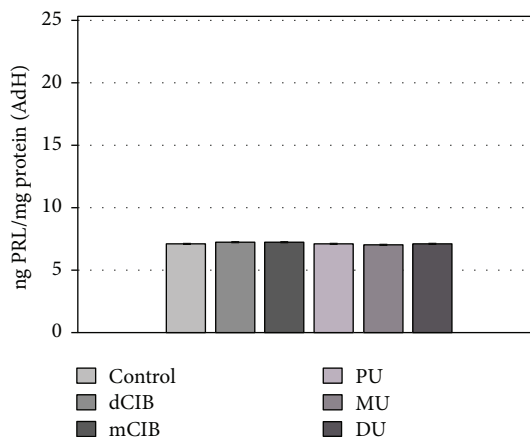


FIGURE 5: Effects of EDC on PRL release in normal rat AdH cultures, *in vitro*. Mean (PRL/prolactin/level) \pm SEM. The mean and SEM are calculated from $n=12$. Abbreviations: dCIB = 1,4-dichlorobenzene: 0.1 ng/ml; mCIB = chlorobenzene mix: 0.1 ng/ml; PU = phenuron: 10^{-6} M; MU = monuron: 10^{-6} M; DU = diuron: 10^{-6} M.

and DU $15,197.58 \pm 4.99$). EDC effects were detected in the regulation model (+B+AVP-feedback) (mean level \pm SEM (pg ACTH/mg protein): control 2195.50 ± 4.69 ; dCIB 2579.42 ± 3.98 ; mCIB 2513.00 ± 2.94 ; PU 2421.00 ± 2.28 ; MU 2553.83 ± 7.87 ; and DU 2690.42 ± 4.14).

Figure 5 shows the PRL release in AdH cultures in the presence of the ED compounds tested. It can be seen that the ED compounds did not trigger relevant differences in PRL release (mean level \pm SEM (ng PRL/mg protein): control: 7.13 ± 0.04 ; dCIB 7.28 ± 0.03 ; mCIB 7.26 ± 0.01 ; PU 7.12 ± 0.02 ; MU 7.02 ± 0.02 ; and DU 7.14 ± 0.01).

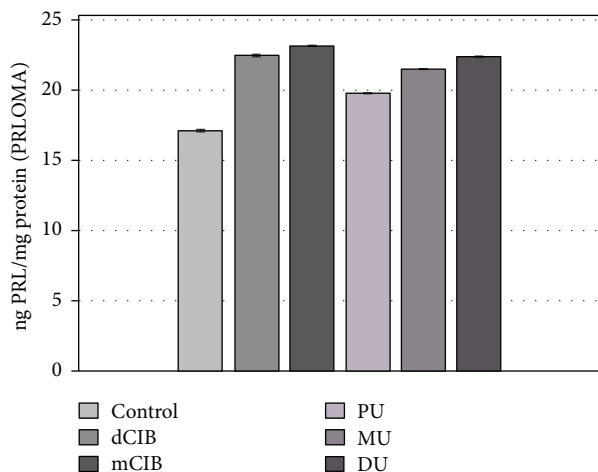


FIGURE 6: Effects of EDC on PRL release in rat PRLOMA cultures, *in vitro*. Mean (PRL/prolactin/level) ± SEM. The mean and SEM are calculated from $n = 12$. Abbreviations: dCIB = 1,4-dichlorobenzene: 0.1 ng/ml; mCIB = chlorobenzene mix: 0.1 ng/ml; PU = phenuron: 10^{-6} M; MU = monuron: 10^{-6} M; DU = diuron: 10^{-6} M. All EDC groups differ significantly from the control ($p < 0.001$) for basal release.

Figure 6 shows the PRL release of rat PRLOMA cultures in the presence of ED chemical agents as described in the experimental protocol. All the examined compounds showed a significant stimulating effect (dCIB: 22.47 ± 0.03 ; mCIB: 23.17 ± 0.02 ; PU: 19.82 ± 0.01 ; MU: 21.50 ± 0.02 ; and DU: 22.41 ± 0.02 ng PRL/mg protein) on the release of PRL compared to that of the control (17.14 ± 0.02 ng PRL/mg protein) in PRLOMA cultures.

4. Discussion and Conclusions

We have studied the effects of PU, MU, DU, dCIB, and mCIB as potential environmental factors, on the basal release of ACTH (Figure 3) (creative element: mobile network junction [49]), which plays a role in the essential functioning of the AdH/AC axis [30] (interpreted in the human homeostasis network as an “AND” function). The ED effects of the applied compounds could be detected, since each chemical agent generated significant changes in the ACTH secretion of PRLOMA cells (Figure 4) in the basal group. PRL release can be increased by enhancing peripheral estrogen levels, which can be the “OR” cycle in connection to ACTH release. In our work, we modeled a homeostatic regulatory neuroendocrine network both under normal regulation (AdH model) and under a neuroendocrine disturbance (+ER). (First, cell cycle change was induced via autoregulatory cell dysfunction [29].) In the PRLOMA cells that were already altered by the control cycle, the level of not only PRL (Figure 6), but also that of ACTH increased (Figure 4). The events of the neuroendocrine regulatory cycles in our investigation suggest that the outlined mechanisms can be regarded as homeostatic biological network elements [50].

The AVP-activated ACTH hormone secretion of AdH cells was significantly increased by all ED compounds used when compared to the control (Figure 3, +AVP group). ED

agents caused increased ACTH release in the AVP-activated PRLOMA cells as well (Figure 4, +AVP group). It is well known that in biological complexities (biological networks), regulations appear as the resultants of activating and inhibiting functions [51]. Both in the normal AdH and PRLOMA cell types, the negative feedback effect of corticosterone was modeled by the inhibition of AVP-activated ACTH release [52]. Figure 3 demonstrates that AdH cells were able to maintain their inhibitory functions despite a treatment with EDC (Figure 3, +B+AVP group). Thus, the adaptive potential of healthy AdH cells in cases of chemical environmental load modeled by EDC remained reversibly regulated. The regulation of PRLOMA cells was incomplete in the presence of EDC (Figure 4, +B+AVP group); although the inhibitory effect of corticosterone on ACTH secretion is also present in PRLOMA, the maintenance of this effect is strongly disrupted by ED compounds (Figure 4). In this context, the results can be interpreted as an environmental disruption leading to irreversible processes. The presence of ED compounds enhanced the already overexpressed ACTH secretion in PRLOMA cells (Figure 4), which was further increased by AVP activation. Therefore, it can be assumed that both the structure and the endocrine cell function of PRLOMA were damaged [53]. Due to the role ACTH plays in essential life functions, this result may have significance in the environmental exposition of prolactinoma patients and in the development of adaptational potential disorders in healthy individuals [54], as ACTH regulation is present in human adaptation as an open dynamic requirement, which is modeled as an “AND” logical function algorithm. Our results show that PRL secretion in normal AdH cells was not influenced by EDC in the applied experimental system (Figure 5). However, the already elevated PRL secretion of PRLOMA cells was further enhanced by EDC (Figure 6).

HTP is defined in relation to dCIB with a standard approach [43] and uniform risk assessment. The authors wished to provide an opportunity for comparison by following dCIB effects too. Accordingly, uron/dCIB relations were determined in AdH and PRLOMA cells. Our results show that the EDC classification of the examined uron compounds strongly approximates the effects of dCIB in an AdH model (Figures 3 and 5). However, basal ACTH release of PRLOMA is more effective in the presence of DU (uron/dCIB \rightarrow 0.97–0.99) than dCIB (+DU/dCIB = 1.05). In baseline PRL secretion, the uron effects on AdH cells approximated those of dCIB (urons/dCIB: 0.96–0.98). In the case of PRLOMA, the EDC effects of the investigated uron compounds on PRL secretion approximated those of dCIB (urons/dCIB: 0.96–0.98). However, in the case of AVP-activated ACTH release in the PRLOMA model, dCIB effect was exceeded by the agents MU and DU (PRLOMA: +AVP + MU/+AVP + dCIB = 1.02; PRLOMA: +AVP + DU/+AVP + dCIB = 1.03).

The HTP values of PRLOMA can be interpreted with double risk classification according to our present study, because when the doses of ED compounds exceed those necessary for irreversible effects (such as suspension of corticosterone inhibition), regulatory dysfunctions may present difficult-to-treat disease processes.

It can be seen from the above how important researching human homeostatic network disorders is when one wishes to evaluate the health consequences of environmental factors.

Data Availability

Requests for data will be considered by the corresponding author.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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