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EFFECT OF *o,p'*-DDD AND Li^+ ON APOPTOTIC DNA FRAGMENTATION IN CONVENTIONALLY NORMAL AND TUMOUR TISSUES OF HUMAN ADRENAL CORTEX

V. M. PUSHKAREV, N. D. TRONKO, N. N. KOSTYUCHENKO, A. S. MIKOSHA

V. P. Komisarenko Institute of Endocrinology and Metabolism, Academy of Medical Sciences of Ukraine, Kyiv; e-mail: endo@i.kiev.ua

The actions of 1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2-dichloroethane (o,p'-DDD), potassium and lithium ions upon apoptotic processes in conventionally normal and tumour tissues of human adrenal cortex were studied. There was no effect of K^+ on the apoptosis in tumour tissue. o,p'-DDD – the specific drug for conservative therapy of adrenocortical cancer – enhanced the apoptotic DNA fragmentation in all tested tissues. The conclusion was made that apoptosis may be involved in curative effect of o,p'-DDD in adrenal cortex.

Lithium ions, which are used in clinic as antidepressant, inhibited the apoptosis in conventionally normal tissue and in most tumours. On the other hand, lithium enhanced the DNA fragmentation in the postoperative tissue of patients with Cushing disease. The possible mechanisms mediating lithium effects on the adrenal cortex are discussed.

Key words: apoptosis, o,p'-DDD, K^+, Li^+, human adrenal tumours.

Apoptosis plays an important role in the living organism, supporting a dynamic equilibrium between proliferation and cell death and thus regulating the cellular mass of organs [1]. Signaling cascades, that initiate apoptosis make up the genetically formed programs represented in the majority, if not all, cells, though in inactive state. Various physiological and stress agents can initiate these apoptotic programs [2]. Disorder of this process causes different pathologies, including malignancy, because of insufficient apoptosis [3].

The elimination of tumour cells is often realized by apoptosis which can be enhanced by some antineoplastic drugs [4]. Among well known antitumour compounds, 1-(*o*-chlorophenyl)-1-(*p*-chlorophenyl)-2,2-dichloroethane (*o,p'*-DDD, mitotane) is one of the most specific compounds. It is the unique agent for conservative therapy of adrenal cancer. We supposed that adrenocorticocyte plasma membrane that is rich in lipids is the target of mitotane action [5]. It is assumed that the generation of free radicals is an important intermediate step in the *o,p'*-DDD action. However, it is unknown whether the cells of the adrenal cortex die through the mechanism of necrosis or apoptosis.

It has long been known that development and metamorphosis of embryos are very sensible to lithium action. Partly, it can point to the possibility of its effect on mitosis and apoptosis. Since the 70's of the last century, the effects of lithium on the endocrine system and, in particular, on adrenals were studied intensively [6]. Although lithium has long been used in psychiatry and endocrinology, the molecular mechanisms of its action are poorly understood. It is considered that lithium affects the second messenger systems. It inhibits the phosphatase of inositol phosphate and thus disturbs transfer of regulatory signals by the polyphosphoinositide messenger system [7]. There is also an evidence that lithium decreases the activity of adenylate cyclase and the level of cAMP in the brain cortex [8], and inhibits some monophosphoesterases [9]. These effects can cause changes in the activity of the corresponding protein kinases and in the pattern of phosphorylated cellular proteins. We showed that in adrenocortical cells lithium inhibited Ca^{2+}/phospholipids-dependent protein kinase (PKC) activated at increased K^+ concentrations in the incubation medium [10]. Lithium also regulated the c-Jun N-terminal kinase (JNK) activity through PKC-dependent mechanisms in the brain [8]. Data on lithium-dependent suppression of glycogen synthase kinase-3β (GSK-3β) involved in the Wnt-signalling were obtained recently [9].

The main goal of this work was to carry out a comparative study of the dependence of apoptotic DNA fragmentation intensity upon *o,p'*-DDD, K^+ and Li^+ action in conventionally normal and tumour tissues of the human adrenal cortex.

Materials and Methods

The study was approved by the Ethics Committee of the Institute of Endocrinology & Metabolism.

Conditions of incubation. Tumours and tissues of the adrenal cortex, adjacent to tumours, were used. The sections of tissue, that kept normal structure, were separated from the tumour tissue and designated as "conventionally normal tissue" (CNT). Pathologists, for refinements of final diagnoses of the disease, studied tissues in parallel. Adrenal tissue was placed on ice, trimmed from fat and connecting tissue; tumour tissue was separated from CNT and both were cut into slices. Slices were incubated for 3 hours at 37 °C in 1 ml of buffer of following composition: 10 mM Na_2HPO_4, 1 mM NaH_2PO_4, 130 mM NaCl, 1.27 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES (pH 7.4), 2 mg/ml bovine serum albumin (BSA). LiCl, *o,p'*-DDD, KCl (to 3.5 or 8.5 mM) were added in test tubes. After the completion of incubation, tubes were cooled quickly and DNA was extracted.

DNA extraction. Tissue was homogenized at +4 °C in glass homogenizer in a buffer that contained 10 mM tris-HCl (pH 7.5), 10 mM EDTA, 0.1 M NaCl. Ratio of tissue : buffer was approximately 1 : 5. Freshly prepared proteinase K (final concentration 0.2 mg/ml) and sodium dodecyl sulfate (1%) were added to the homogenate. Incubation was performed at 60 °C for 15–20 min, then overnight at 45 °C. RNAse solution (10 mg/ml), prepared preliminary and preincubated for 15 min at 90 °C for DNAse inactivation, was added to homogenate up to the concentration of 0.1–0.2 mg/ml, and the incubation was prolonged for 1 h at 37 °C. Then NaCl solution (5 M) was added to the concentration of 0.5 M and the sample was deproteinized by 2 volumes of the mixture chloroform : isoamyl alcohol (24 : 1). The water phase was withdrawn and deproteinization repeated up to disappearance of the interphase. DNA was precipitated by 3 volumes of cold ethanol overnight at -18 °C. High molecular DNA was picked up by a glass stick, transferred in a separate tube, dried for 10 min and dissolved in a homogenization buffer without NaCl (TE-buffer). The remaining DNA was centrifuged at 30 000 g in the SW-55Ti rotor (Spinco L5-50) at 2 °C for 15 min. The pellet was washed by 80% ethanol, centrifuged once again, dried in the air for 10 min and dissolved in TE-buffer. DNA concentration and quality were estimated using a spectrophotometer "Jenway" (model 6405) at 254 nm wavelength.

DNA electrophoresis in agarose gel. 2.25% agarose gel was prepared on a buffer containing 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0 (TBE-buffer). Ethidium bromide at 1 μg/ml was added in gel and buffer. Samples were prepared by mixing up the DNA solution with bromphenol blue (0.025%) and sucrose (45%). 5 μg of DNA were applied per each well. The volume of the sample did not exceed 20 μl. A wide range (50–10 000 base pairs) DNA markers were used. Electrophoresis was carried out for 2 h at 100 V. Gels were photographed by a digital video camera, scanned using "Scion Image" software, and intensity of the DNA fragmentation was estimated. The quantity of four last fragments (190–760 bp) of DNA in each gel track was referred to total DNA quantity in this track taken as 100 %.

Reagents. All the salts, HCl, NaOH were provided by «Merck» (Germany); HEPES, EDTA, proteinase K, BSA (V fraction, content of lipids less than 0.1%) – were from «Serva» (Germany). Agarose, H_3BO_3, Tris – from «Sigma» (USA). *o,p'*-DDD was synthesized in the laboratory of organic synthesis and reagents of the Institute. The rest of the reagents were provided by local suppliers. All solvents were distilled before use.

Statistics. Student's paired *t*-test and Wilcoxon U-test were used.

Results and Discussion

Determination of DNA fragmentation, which indicates the onset of the final phase of apoptosis, is one of the most reliable estimations of apoptotic processes extension. Transformation of chromatin is an important stage of programmable cell death. In this case DNA is cleaved by endonucleases into fragments of fixed size, unlike at necrosis, when DNA is disintegrated into low molecular weight products. We used the process of DNA fragmentation for determining the intensity of apoptotic processes in CNT and tumour tissues of human adrenals. In some cases the caspase-3 activity was determined as an additional control of apoptosis [11].

In preliminary experiments we determined the dependence of DNA fragmentation degree in tissue of human adrenal glands on the incubation duration. The incubation for 1 or 2 hours in conditions described in Methods did not cause DNA fragmentation. Beginning from 3 h of incubation, clear DNA fragmentation was observed in CNT and in tumour tissue both in the control and experimental conditions. The prolongation of incubation caused an insignificant increase of DNA fragmentation; therefore, we incubated tissue for 3 h.

Effect of o,p'-DDD on the degree of DNA fragmentation in tumours of human adrenals. Apoptosis plays an important role in the elimination of tu-

mour cells and increases in them under the effect of some antitumour drugs [4]. Since *o,p′*-DDD is used for treatment of certain types of adrenal malignant tumours, the task of this work was to study the effect of mitotane, as well as the combined effect of potassium ions and *o,p′*-DDD, on apoptosis in the tumour tissues of human adrenals. It was expected that there would be intensification of apoptotic processes in tumours of the adrenal cortex in the presence of *o,p′*-DDD.

Fig. 1 presents the results of densitometric analysis of electrophoresed DNA extracted from the tumour tissue (corticosteroma) after incubation at a physiological concentration of potassium (3.5 mM). The incubation of tissue with *o,p′*-DDD significantly enhanced apoptotic processes in the tissue (Fig. 1,*2*). An increase of potassium ions concentration in the incubation medium did not cause any noticeable changes of apoptosis in this tumour (Fig. 1,*1* and 1,*3*). The incubation of adrenocortical tissue with *o,p′*-DDD in the incubation medium which contained 8.5 mM K^+, as well as in the medium with 3.5 mM K^+ (Fig. 1,*2* and 1,*4*) resulted in considerable enhancing of DNA fragmentation. Apparently the tumour cell is protected from proapoptotic action of potassium ions, and apoptosis can be induced by more competent stimuli such as *o,p′*-DDD.

Thus, the data presented show that the incubation of slices of the adrenal cortex tumour tissue with *o,p′*-DDD considerably strengthened DNA fragmentation. Probably, the antitumour effect of this drug involves the induction of apoptosis in tumour cells.

Dependence of DNA fragmentation extent in CNT and tumour tissue of human adrenals on lithium ions concentration in the incubation media. There are numerous evidences of the antiapoptotic action of lithium ions in the nervous tissue [11]. In our studies lithium manifested the antiapoptotic effect in adenoma (adenocarcinoma) and CNT, adjacent to adenoma (Fig. 2 and 3). It can be seen from electrophoregram that a rise in Li^+ concentration in the incubation medium results in a clear decrease in DNA fragmentation with a simultaneous increase in the fraction of high-mo-

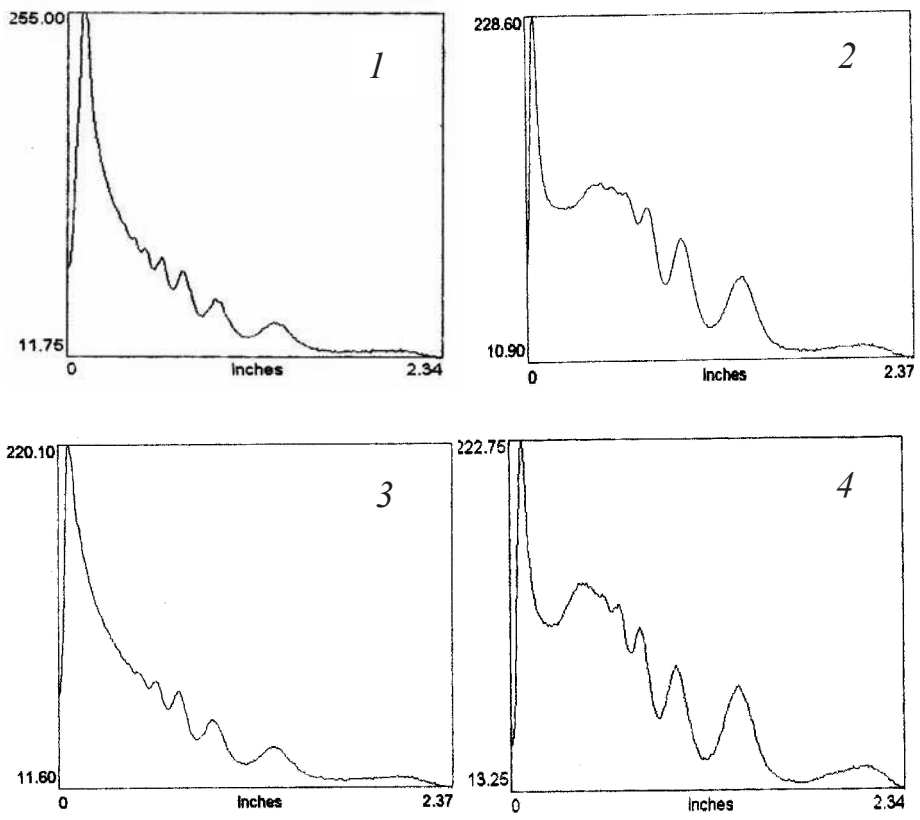

Fig. 1. Densitograms of agarose gels with fragments of DNA from tumour tissue of human adrenal cortex (corticosteroma). Absciss – distance from start point, ordinate – optical density of DNA bands in conventional units. 1 – tissue incubated at 3.5 mM of K^+ (control); 2 – tissue incubated at 3.5 mM of K^+ with o,p′-DDD (50 μM); 3 – tissue incubated at 8.5 mM of K^+; 4 – tissue incubated at 8.5 mM of K^+ with o,p′-DDD (50 μM); Figures 1 and 2 show representative examples of 3 experiments.

lecular DNA (Fig. 2). This observation confirms the existing data which show that lithium inhibited apoptosis and expressed a well-defined neuroprotective effect [12–15]. It was assumed that the effect upon Wnt-signaling mechanisms is the basis of antiapoptotic effect of lithium, via GSK-3β suppression [12, 13]. GSK-3β is negatively regulated by the PI3K/Akt cascade and, in turn, inhibits β-catenin by phosphorylation. The unphosphorylated β-catenin migrates into the nucleus, where it activates the transcription factors LEF and TCF which transactivate genes participate in growth processes [16]. PI3K activation by lithium ions results in an increase in neuronal calcium concentration, presumably as a result of phospholipase C activation and IP_3 formation [14], that can activate cell survival processes. In addition, lithium activates MAP kinases, which also cause cell survival [12]. The antiapoptotic effects of lithium can also be associated with AP-1 transcription factor,

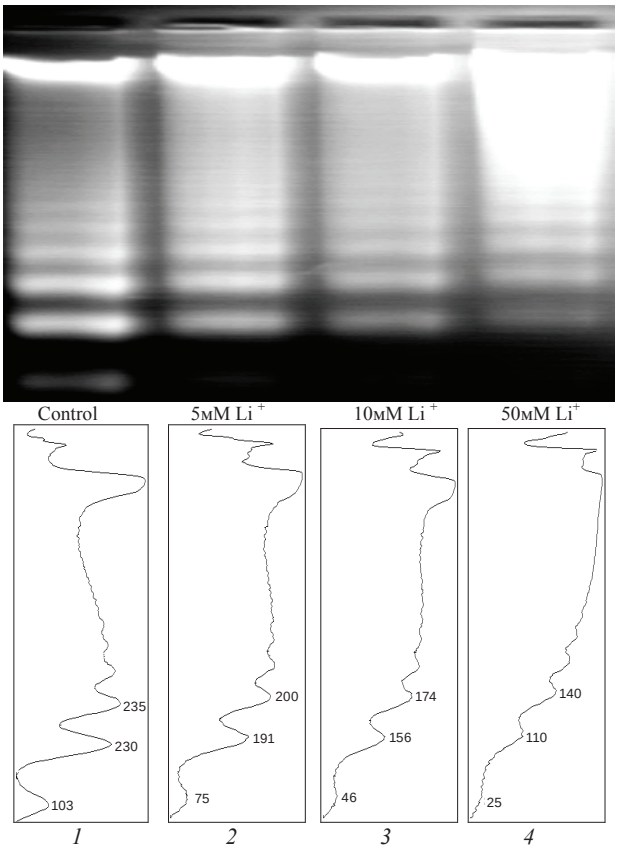

Fig. 2. Electrophoregram of DNA from CNT adjacent to adenoma (adenocarcinoma). 1 – control; 2 – Li^+ (5 mM); 3 – Li^+ (10 mM); 4 – Li^+ (50 mM). The densitograms of corresponding gel tracks are presented on the inferior panel. Absciss – optical density of DNA bands in conventional units, ordinate – distance from the start (upper) point.

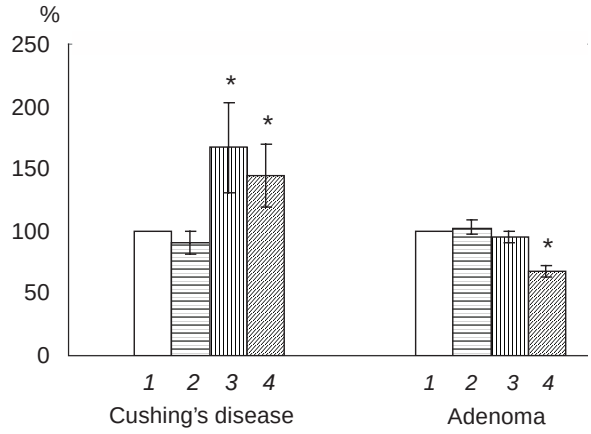

*Fig. 3. Comparison of DNA fragmentation (for last 4 fragments – 190–760 bp) in tumour and hyperplasic tissue of human adrenal cortex after incubation at different Li^+ concentrations. 1 – control; 2 – Li^+ 5 mM; 3 – Li^+ 10 mM; 4 – Li^+ 50 mM. As 100% was adopted the DNA fragmentation in control. M ± m; n = 5 (Cushing's desease), n = 3 (adenoma); * significantly different from control (U-test), P < 0,05.*

protein binding to a cAMP-responsive element (CREB) and proteins of Bcl2 family [12]. Blocking of c-Jun-signaling pathways by lithium, related to proapoptotic mechanisms, which can regulate the expression of the proapoptotic Bim in a tandem with GSK-3β, was described [13]. Data on activation of NFκB transcription factor and MAP kinases p38 and ERK1/2 by lithium were obtained as well [15]. Lithium enhanced NFκB binding to DNA and its transcription activity, which caused the activation of interleukin-8 mRNA expression. In addition, NFκB is able to increase the expression of apoptosis inhibitors of IAP family proteins [17].

Unlike adenoma, apoptosis enhancement in Cushing adrenocortical tissue in the presence of high concentrations of lithium was observed (Fig. 3). Although most data point out the antiapoptotic effects of lithium, there are also arguments in favour of proapoptotic effects of this ion [18–20]. As a rule, lithium increased apoptosis initiated through TNF-R superfamily death receptors [18, 20]. Thus, lithium enhanced TRAIL-induced apoptosis in the prostate cancer cells [18]. Similarly to antiapoptotic action, the proapoptotic effect of lithium can be related to activation of AP-1 and c-Jun transcription factors [19]. The authors suppose that the proapoptotic effect of lithium is mediated by protein kinase C, although most data [7, 8, 10] evidenced for the inhibition of PKC activity by lithium. It is also known that

PKC usually mediated antiapoptotic processes and proliferation [21].

Thus, data on the lithium effects in different tissues and the mechanisms of these effects are contradictory. Probably, the target tissue type (tumours) and experimental conditions determine the direction of these effects. It has been concluded, that the use of lithium for treatment of human depression and necessity of search for possibilities of selective defence of normal tissue with simultaneous strengthening of apoptosis in specific tumour makes Li^+ the promising agent in the experimental oncology.

ВПЛИВ *o,n′*-ДДД ТА Li^+ НА АПОПТИЧНУ ФРАГМЕНТАЦІЮ ДНК В УМОВНО НОРМАЛЬНІЙ І ПУХЛИННІЙ ТКАНИНАХ КОРИ НАДНИРКОВИХ ЗАЛОЗ ЛЮДИНИ

В. М. Пушкарьов, М. Д. Тронько, Н. М. Костюченко, О. С. Микоша

Інститут ендокринології та обміну речовин ім. В. П. Комісаренка, Академія медичних наук України, Київ;
e-mail: endo@i.kiev.ua

Вивчали дію *o,n′*-дихлордифенілдихлоретану (*o,n′*-ДДД), іонів літію та калію на апоптичні процеси в пухлинах та умовно нормальній тканині кори надниркових залоз. Іони калію не впливали на апоптоз у пухлинній тканині. Специфічний засіб консервативної терапії раку надниркових залоз – *o,n′*-ДДД – посилював апоптичну фрагментацію ДНК в усіх досліджених тканинах. Висловлено гіпотезу, що терапевтична дія *o,n′*-ДДД на кору надниркових залоз пов'язана з апоптозом.

Іони літію, який використовується в клінічній практиці як антидепресант, пригнічували апоптоз в умовно нормальній тканині та більшості пухлин. З іншого боку, у тканині хворих на хворобу Іценка–Кушинга літій посилював фрагментацію ДНК. Обговорюються можливі механізми, що опосередковують дію літію на кору надниркових залоз.

Ключові слова: апоптоз, *o,n′*-ДДД, K^+, Li^+, пухлини надниркових залоз людини.

ВЛИЯНИЕ *o,n′*-ДДД И Li^+ НА АПОПТИЧЕСКУЮ ФРАГМЕНТАЦИЮ ДНК В УСЛОВНО НОРМАЛЬНОЙ И ОПУХОЛЕВОЙ ТКАНЯХ КОРЫ НАДПОЧЕЧНИКОВ ЧЕЛОВЕКА

В. М. Пушкарев, Н. Д. Тронько, Н. Н. Костюченко, А. С. Микоша

Институт эндокринологии и обмена веществ им. В. П. Комиссаренко, Академия медицинских наук Украины, Киев;
e-mail: endo@i.kiev.ua

Изучали действие *o,n′*-дихлордифенилдихлорэтана (*o,n′*-ДДД), ионов лития и калия на апоптические процессы в опухолях и условно нормальной ткани коры надпочечников. K^+ не влиял на апоптоз в опухолевой ткани. Специфическое средство консервативной терапии рака надпочечников – *o,n′*-ДДД – усиливал апоптическую фрагментацию ДНК во всех исследованных тканях. Делается вывод, что терапевтическое действие *o,n′*-ДДД на кору надпочечников связано з апоптозом.

Ионы лития, который используется в клинической практике как антидепрессант, угнетали апоптоз в условно нормальной ткани и большинстве опухолей. С другой стороны, в ткани больного болезнью Иценко–Кушинга литий усиливал фрагментацию ДНК. Обсуждаются возможные механизмы, опосредующие действие лития на кору надпочечников.

Ключевые слова: апоптоз, *o,n′*-ДДД, K^+, Li^+, опухоли надпочечников человека.

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