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EFFECTS OF HYDROCORTISONE AND INSULIN ON DNA INTERNUCLEOSOMAL FRAGMENTATION IN RAT THYMOCYTE NUCLEI

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In the present work the effect of insulin and hydrocortisone on internucleosomal DNA fragmentation, the well known hallmark of apoptosis, in isolated rat thymocyte nuclei after 4 and 24 hours of hormones in vivo action has been examined. The changes of enzyme activity of poly(ADP-ribose)polymerase-1 (PARP-1) concomitantly were investigated.

It was shown, that in 4 hours of hydrocortisone in vivo action no significant alterations of DNA oligonucleosomal fragmentation were observed. However, after 24 hours of steroid administration, sharp increase in fragmentation intensity and pattern were observed in thymocyte nuclei. Peptide hormone insulin had a different effect on DNA cleavage. In contrast to hydrocortisone, it significantly decreased the intensity of fragmentation in time-dependant manner. The data revealed that in 4 hour of both hormones action there were no changes in PARP-1 activity. Nevertheless, the activity of the enzyme declined about two-fold in 24 hours of hormones administration, which can probably reflect non-specific cell response upon hormonal stress.

Hydrocortisone – Insulin – PARP-1 activity – DNA fragmentation

Ներկայացված աշխատանքում ուսումնասիրվել է ինսուլինի և հիդրոկորտիզոնի ազդեցությունը հորմոնների ներարկումից 4 և 24 ժամ հետո թիմոցիտների մեկուսացված կորիզներում ԴՆԹ-ի ինտերնուկլեոսոմային ֆրագմենտավորման վրա: Զուգահեռ ուսումնասիրվել է նաև հորմոնների ազդեցությունը պոլի(ԱԴՖ-ռիբոզ)պոլիմերազի (ՊԱՌՊ-1) ակտիվության վրա:

Ստացված արդյունքները ցույց են տալիս, որ հիդրոկորտիզոնի *in vivo* ազդեցությունից 4 ժամ հետո ԴՆԹ-ի ճեղքավորման զգալի փոփոխություններ տեղի չեն ունենում: Սակայն հորմոնի ներարկումից 24 ժամ հետո կտրուկ մեծանում է թիմոցիտների կորիզներում ԴՆԹ-ի օլիգոնուկլեոսոմային ճեղքավորման ինտենսիվությունը և փոխվում է բնույթը: Ինսուլինը ցուցաբերում է հիդրոկորտիզոնին հակադիր ազդեցություն: Կախված ինսուլինի in vivo ազդեցության տևողությունից, զգալիորեն ճնշվում է ԴՆԹ-ի ինտերնուկլեոսոմային ֆրագմենտավորումը: Արդյունքները վկայում են, որ հորմոնների *in vivo* ազդեցությունից 4 ժամ հետո կորիզներում ՊԱՌՊ-1-ի ակտիվությունը չի փոխվում, իսկ 24 ժամ հետո, ֆերմենտի ակտիվությունը նվազում է մոտ 2 անգամ, ինչը կարող է վկայել բջիջների հորմոնային սթրեսի նկատմամբ ոչ սպեցիֆիկ պատասխանի մասին:

Հիդրոկորտիզոն –ինսուլին –ՊԱՌՊ-1-ի ակտիվություն –
ԴՆԹ-ի ֆրագմենտավորում

В представленной работе исследовано действие инсулина и гидрокортизона на интернуклеосомальное расщепление ДНК в изолированных ядрах тимоцитов через 4 и 24 ч после введения гормонов животным. Параллельно изучалось действие гормонов на активность поли(АДФ-рибоз)полимеразы 1 (ПАРП-1).

Полученные результаты показывают, что заметных изменений в интернуклеосомальном расщеплении ДНК через 4 ч после инъекции гидрокортизона не происходит. Однако через 24 ч интенсивность олигонуклеосомального расщепления ДНК возрастает и меняется его характер. Инсулин проявляет действие, противоположное гидрокортизону. В зависимости от длительности *in vivo* действия инсулина интернуклеосомальное фрагментирование ДНК заметно подавляется. Итак, через 4 ч действия обоих гормонов активность ПАРП-1 в ядрах не меняется, а через 24 ч действия как одного, так и другого гормона активность фермента уменьшается в два раза, что может свидетельствовать о неспецифическом клеточном ответе на гормональный стресс.

Гидрокортизон – инсулин – активность ПАРП-1 – фрагментация ДНК

Apoptotic cell death is an essential physiological process that regulates the normal development of the immune system by selective elimination of unwanted thymocytes. The final and “point of no return” phase of apoptosis is the DNA oligonucleosomal fragmentation which results in effective DNA degradation and their further elimination by phagocytes [1,2]. During the development and differentiation, T cells have to undergo a rigorous selection process in the thymus. It is widely recognized, that a tight cross-talk between the immune and neuroendocrine systems is well developed in thymus. Particularly, the physiological processes and tissue homeostasis of the thymus are modulated by a range of biological circuits including those mediated by peptide and steroid hormones [3].

It was previously shown that insulin-like growth factor (IGF)/insulin family and epidermal growth factor (EGF) are capable of stimulating the growth of various cell types by signaling through their corresponding tyrosine kinase receptors. On the other hand, it is known, that both classes of growth factors and their receptors can prevent apoptosis in many cells and cell types. In addition to its well-known contribution to carbohydrate, fat and protein metabolism, insulin can also regulate other key biological processes e.g. cell survival, apoptosis, proliferation and cell cycle in general [4]. In thymocytes, insulin is known to counteract the well established proapoptotic effect of hydrocortisone – profound member of glucocorticoid (GCs) family [5].

Glucocorticoids are steroid hormones which are considered to have prominent role in regulating key processes in the development of the immune system. It is well known that their synthetic agonists are one of the most widely prescribed classes of drugs in the world and are indispensable in the treatment of autoimmune diseases, inflammatory disorders and cancer [6]. In the last case, glucocorticoids and their synthetic counterparts are often used not only as supplementary agents, but also as chemotherapeutic drugs in the threatment of thymomas. In the contrast to widespread knowledge of the role of hydrocortisone in thymocyte apoptosis, the molecular mechanisms of its action are still elusive [7].

One of the key factors which mediates the cross-talk of various regulatory death pathways is poly(ADP-ribose)polymerase-1 (PARP-1). The activity of latter is tightly regulated by different exogenous and endogenous cell signals [8]. Coming from this, and considering the powerful signaling role of insulin and hydrocortisone, the activity of PARP-1 in nuclei isolated from thymocytes after 4 and 24 hours of hormones injection was examined.

Materials and methods. All the chemicals and reagents were purchased from Sigma. Outbred, 4-6 weeks old, white, male rats (100 grams of body weight) were used throughout experiments. Hydrocortisone was injected intraperitoneal 50 mg/kg animal weight, insulin was administrated after 15 minutes of glucose injection (approx. 0.12% in blood) intraperitoneal 20 units per 1 kg animal weight in water solution.

After 4 and 24 hours of hormone injection animals were decapitated and nuclei of thymocytes were isolated by the method of [9]. Sucrose solutions buffered with 25 mM Tris (pH 7.5) containing 150 mM NaCl, 60 mM KCl, 15 mM spermine and 5 mM spermidine were used. DNA preparation and electrophoresis procedures were carried by methods described elsewhere [10]. The relative amounts of oligonucleosomal fragments in each sample were calculated using special software "Image Gauge V4.0" for densitometric analysis of electrograms.

PARP-1 activity assay was based on chemical quantitation on NAD^+ in PARP-1 buffer [11]. The assay was modified for NAD^+ detection in isolated nuclei by us. Briefly, 2000 µl of isolated nuclear suspensions containing 1000 µg DNA were incubated with $CaCl_2$, $MgCl_2$ (final concentrations of 1 and 6 mM respectively) and 0.5 mM NAD^+ for 10 minutes. The reaction was stopped by removal of nuclei from reaction mixture by centrifugation at 13000 g for 2 minutes. Supernatant was aliquoted to 700 µl in new test tubes to perform NAD^+ quantitation. NAD^+ content in aliquots was determined by sequential addition of 2 M KOH, 20% acetophenone (in EtOH) and 88% formic acid yielding final concentrations of these agents according to original assay. PARP-1 assay buffer was used as a reference containing 0.5 mM NAD^+ and all aforementioned reagents with corresponding final concentrations. NAD^+ quantity was measured by the absorbance of NAD^+ reaction product at wavelength 378 nm. The activity of PARP-1 was defined as the reduction of NAD^+ content in the samples which was determined by subtracting the absorbance of test samples from the reference.

Results and Discussion. DNA internucleosomal fragmentation is one of the major hallmarks of apoptosis which is executed by various apoptotic endonucleases. The changes in the fragmentation pattern characterize the level of chromatin compaction in nuclei which is known to be affected by various intranuclear and extracellular factors. Taking into consideration this, the influence of hydrocortisone and insulin hormones on DNA oligonucleosomal degradation after 4 and 24 hours of hormone actions was assessed.

The obtained results show that in 4 hours of in vivo action of hydrocortisone no significant effect was observed in the dynamics and strength of DNA internucleosomal cleavage. As in the control nuclei, it started after 6 hours of nuclei incubation and gradually increased up to 24 hours (Fig. 1). In contrast to hormone' shortterm action (4 hours), after 24 hours of hormone administration, marked DNA fragmentation was observed, which shows that the process had already begun in the nucleus before the isolation procedure (i.e. *in vivo*). The amounts of DNA fragments longer than 1000 b.p. in length were assessed by Image Gauge V4.0 software applied to DNA electrograms.

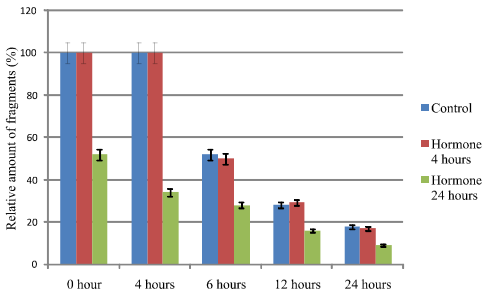

Fig. 1. Relative amounts of total DNA fragments (more than 1000 b.p.) in thymocyte nuclei after 4 and 24 hours of in vivo hydrocortisone action. Isolated nuclei from the thymocytes of control and hormone administered rats were kept at 37°C in the incubation medium for 0, 4, 6, 12 and 24 hours.

Previously similar results were obtained based on experiments using adrenoectomised animals or cell cultures. Our results clearly demonstrate, that the DNA fragmentation can be induced by hydrocortisone not only in adrenoectomised animals or cell cultures but also in young rats that did not undergo any surgery [12].

The goal of the next set of experiments was the evaluation of insulin effect on DNA fragmentation in thymocyte nuclei. The results summarized in Fig. 2 indicate that after insulin administration the internucleosomal fragmentation of DNA is significantly inhibited in time-dependant manner. In 4 and 24 hours of hormone in vivo action the total amount of DNA fragments bigger than 1000 b.p. were increased about 2- and 3-fold respectively (Fig. 2).

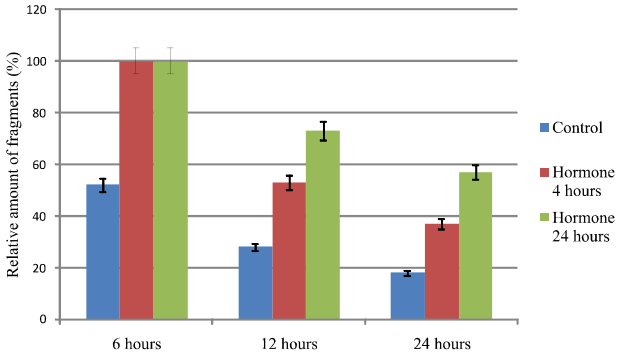

Fig. 2. DNA oligonucleosomal Relative amounts of total DNA fragments (grater than 1000 b.p.) in thymocyte nuclei after 4 and 24 hours of in vivo insulin action. Isolated nuclei of control and hormone administered rats were kept at 37^{o}C in the incubation medium for 0, 4, 6, 12 and 24.

It is clear that insulin has a strong contrary effect on DNA fragmentation on thymocyte nuclei compared with hydrocortisone.

It is evident that the intensity of DNA cleavage depends upon two main parameters: the level of chromatin condensation (the substrate accessibility) and endonuclease activity responsible for DNA fragmentation in nuclei. Previously is was clearly shown by Boulares and coworkers, that poly(ADP-rybosil)ation by PARP-1 can regulate the activities of main nuclear endonucleases (CAD and DNase γ) by post-translational modifications [13,14]. Considering this, we studied whether insulin and hydrocortisone can affect PARP-1 activity thymocyte nuclei after in vivo treatment.

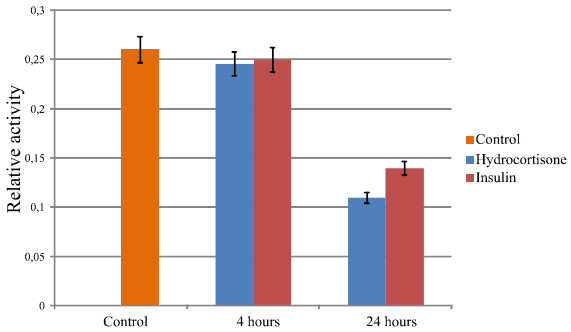

Fig. 3. Changes of PARP-1 activity (relative units) in isolated thymocyte nuclei after 4 and 24 hours of hydrocortisone and insulin injection.

The obtained results show, that in 4 hours of hormone in vivo action, neither insulin nor hydrocortisone had any significant effect on PARP-1 activity in thymocyte nuclei (Fig. 3). However, the activity of the enzyme was about two-fold lower in nuclei, isolated after 24 hours of insulin and hydrocortisone administration.

Considering distinct signaling pathways of steroid and peptide hormones and their different effects on chromatin associated processes, it was unexpected to find that PARP-1, well-established nuclear sensor of chromatin structure, was affected by both hormones in the same manner. We suppose that non-specific original stress-response pathways may be involved in this special case.

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