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ARTICLE *in* NEUROSCIENCE AND BEHAVIORAL PHYSIOLOGY · JULY 1986

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NUCLEASE SENSITIVITY AND METHYLATION OF LIVER CHROMATIN DNA IN RATS IN INITIAL PERIOD OF EFFECT OF GLUCOCORTICOIDS*

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UDC 612.35.014.46:577.175.53]-08

An important task in contemporary molecular endocrinology is the decoding of molecular mechanisms of hormonal regulation of the effect of genes. According to literature data in recent years, steroid hormones, including glucocorticoids, exert their basic effect on transcription only after integration with receptor proteins and association of steroid receptor complexes with specific DNA sites in chromatin [1-5]. In addition, reactivity of a gene in relation to hormone can and should depend not only on primary DNA sequence and the presence of specific receptor-binding sites, but also on higher levels of DNA and DNP predetermined by the overall organization of chromatin. Therefore, it is important to clarify which levels of chromatin organization undergo change or, to the contrary, do not change the character of its organization under the influence of steroid hormones.

Among indices characterizing the structural organization of chromatin, sensitivity of DNA in its structure to DNase I and the character of methylation (quantity and distribution of 5-methyl cytosine) of this DNA have been described in detail. It has been established that transcriptionally active loci and loci of a genome potentially active in transcription display increased sensitivity to DNase I [6] and a reduced level of methylation of cytosine residues [7].

In the present work we analyzed the sensitivity of chromatin in isolated nuclei of rat liver to DNase I and the level of methylation of DNA at the initial stage of exposure to glucocorticoid hormones. It was established that the sharp intensification of transcription induced by glucocorticoids is not accompanied by such conformational reorganizations of chromatin which would change its sensitivity to DNase I; also, no essential changes occurred in level of methylation of DNA.

MATERIALS AND METHOD

White noninbred male rats weighing 140-160 g were used. Bilateral adrenalectomy was performed 3-4 days before administration of hormone; throughout this entire period, rats drank a 0.9% NaCl solution. Hydrocortisone (Richter Company, Hungarian People's Republic) was injected intraperitoneally at 5 mg per 100 g of weight. Activity of tryptophan oxygenase (L-tryptophan: oxygen 2,3-oxidoreductase, E.C. 1.13.11.11) was determined according to Schütz and Feigelson [8]. Hepatocytes were removed from connective tissue elements by forcing liver tissue through a perforated metal plate. Cell nuclei were purified by passage through a solution of dense sucrose, as described previously [9]. The number of nuclei and degree of their purification were determined microscopically. Concentration of DNA in nuclei and in chromatin fractions after washing out the sucrose was measured according to Spirin [10], Burton [11], or according to UV absorption of lysate in a solution of 1% sodium dodecyl sulfate using the empirical formula:

$$C_{DNA}\ (mg/ml) = (A_{260} - A_{300}) \cdot 32.$$

All methods listed produced similar results. Treating nuclei with DNase I (Serva Company, $2 \cdot 10^6$ units of activity/mg) was performed in an ice bath for 1 h at various concentrations of

*We express our gratitude to Prof. V. B. Rozen for his interest in the work and useful comments and to V. V. Ashapkin for his assistance in conducting the individual experiments.

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0097-0549/86/1604-0285$12.50

enzyme. For this 0.5 ml buffer (0.25 M sucrose, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, 30 mM NaCl, 0.01 M Tris-HCl buffer, pH 8.3 at 0°C) containing various concentrations of enzyme was added to 0.5 ml suspension of nuclei (about 100 μg nuclear DNA). In individual cases chromatin was hydrolyzed at 37°C for varying lengths of time at an enzyme concentration of 5 μg/ml. In order to determine activity of endogenous nucleases, nuclei were incubated in parallel in the same conditions without the addition of DNase I. After completion of incubation, samples were centrifuged in the cold for 10 min at 800g. Supernatant containing lightly hydrolyzed fraction of chromatin (Chr I) was removed, and sediments were washed with 1 ml TE buffer (0.01 M Tris-HCl buffer, pH of 8.5, 3 mM EDTA) and incubated at 0°C for 1 h. Then, the suspension was centrifuged in the same regime, removing chromatin in solution (Chr II) from chromatin in the sediment (Chr III). Content of DNA was determined in supernatants and in sediments.

RESULTS AND DISCUSSION

It is generally recognized at the present time that the physiological effect of steroid hormones on a target tissue is governed first of all by change in intensity of synthesis of specific RNA and enzyme proteins in a cell. In particular, in rat liver glucocorticoids induce synthesis of enzymes of gluconeogenesis and amino acid catabolism, one of which is tryptophan oxygenase. Data concerning activity of this enzyme after a single intraperitoneal injection of hydrocortisone are presented in Fig. 1. Administration of hormone leads to substantial increase in activity of tryptophan oxygenase in the liver with the maximum approximately 6 h after injection. These results agree with data of other authors who have investigated activity of tryptophan oxygenase and other induced enzymes in liver [12, 13]. It has been shown that increase in activity of these enzymes is caused by synthesis of them on newly formed matrix RNA [12, 13]. Nuclear synthesis of RNA in liver cells is very sensitive in relation to glucocorticoid hormones. In particular, even 3-4 h after injection of hormone, rate of synthesis of total RNA in hepatocytes increases by two times [14, 15]. In a number of works, clear structural reorganizations of chromatin have been described which accompany hormone-dependent intensification of transcription activity of nuclei. In particular, increase in the proportion of euchromatin [16, 17], increase in the accessibility of chromatin DNA to heterologous RNA-polymerase [18] and to basic stains and fluorochromes [17, 19], and also redistribution of nucleotide sequences between chromatin fractions [20] have been noted. On the other hand, a report has recently appeared that injection of glucocorticoids to rats does not change sensitivity of liver chromatin to exogenous nucleases (micrococcal nuclease and DNase I) [19]. However, these data were not sufficiently convincing, since treatment of nuclei with nucleases was conducted in rather rigid conditions (37°C, appearance of marked proportion of acid-soluble material), which could extinguish possible small differences manifested at the initial stage of chromatin hydrolysis. In addition, incubation of chromatin at 37°C creates favorable conditions for the operation of endogenous nucleases, activity of which can be subject to the influence of hormones. Therefore, we undertook an investigation of the sensitivity of chromatin to DNase I in mild conditions (at 0°C), with division of chromatin into three main fractions (see Materials and Method). For comparison with control (also adrenalectomized) rats, rats analogous in weight were used 3.5 h after administration of hydrocortisone. During this period, rise in activity of inducible enzyme (tryptophan oxygenase) was already rather high (see Fig. 1); even clearer should be an increase in synthesis of mRNA, which precedes synthesis of inducible enzymes [12, 13]. Additional tests showed that during this period of hormonal induction, suppression of DNA synthesis by 70-75% is noted in liver cells. Therefore, the interval of time chosen after administration of hormone corresponds to distinct change in functional status of liver chromatin. However, in the process we could not detect noticeable structural changes in chromatin, tested using DNase I (Fig. 2). It is known that DNase I preferentially hydrolyzes active chromatin, although in a number of cases increased sensitivity to DNase I of individual genes is retained after transcription of them stops [6]. In our tests fraction Chr I is the most accessible to the effect of DNase. Fraction Chr II, extracted by hypotonic solution with EDTA, is also sensitive to DNase I, but to a smaller degree. With increase in concentration of enzyme added to nuclei, the proportion of Chr I increases progressively, while the proportion of Chr II, on the other hand, diminishes, beginning with a concentration of DNase I of about 10 μg/ml (see Fig. 2). With subsequent increase in concentration of DNase I, total yield of hydrolyzed chromatin does not rise, i.e., only part of nuclear DNA is accessible to the effect of the enzyme. Such tests, performed repeatedly (mean error for measurement of yield of nuclear DNA is 1-2%), showed that with hormonal induction such conformational changes do not occur in chromatin which would noticeably change its sensitivity to DNase I as tested in mild conditions.

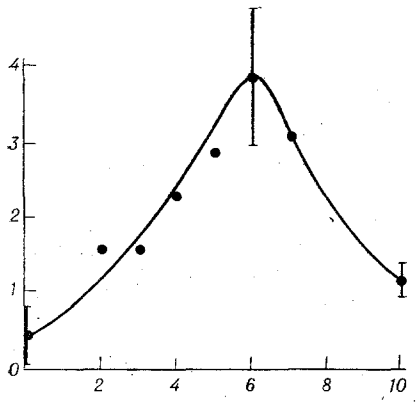

Fig. 1

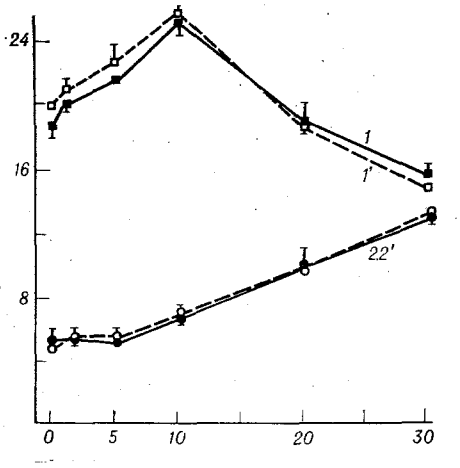

Fig. 2

Fig. 1. Kinetics of induction of tryptophan oxygenase activity in liver after injection of hydrocortisone to rats in a dose of 5 mg per 100 g of live weight. Activity of tryptophan oxygenase was determined spectrophotometrically in temperature-controlled vessels at 37°C according to rise in absorption at 360 nm [8]. Along the abscissa is time after administration of hormone (in h); along the ordinate is enzyme activity (in arbitrary units per 1 mg cytosol protein; ΔA_{360}/min per 1 mg protein).

Fig. 2. Dynamics of hydrolysis by DNase I of rat liver chromatin with differing hormonal status. Incubation of nuclei with DNase I was conducted for 1 h at 0°C. Open circles and squares are control (adrenalectomized) rats; black are analogous rats 3.5 h after administration of hydrocortisone; 1, 1') (Chr I) fractions; 2, 2') (Chr II) fractions. Along the ordinate is yield of DNA (in %); along the abscissa is concentration of DNase I (in μg/ml).

TABLE 1. Level of Methylation of DNA of Rat Liver with Differing Hormonal Status

Group of rats	Level of methylation of cytosine residues* $\frac{m^5C}{C+m^5C}\cdot 100\ \%$
I. Intact animals	3,9±0,2
II. Rats 3-4 days after bilateral adrenalectomy	3,8±0,2
III. Adrenalectomized rats 3.5 h after administration of hydrocortisone	3,9±0,3

*$\bar{M} \pm \sigma$, n = 6; m^5C) 5-methylcytosine; C) cytosine.

It is known that content and distribution of the minor base of 5-methylcytosine series is an important characteristic of the functional state of individual loci of the genome [7]. In particular, undermethylation of individual DNA sites is a permissive factor allowing expression of inducible genes under the influence of hormones and other effectors [21, 22]. In our investigations in parallel with testing of sensitivity of nuclear DNA in chromatin in DNase, we also determined the level of methylation of cytosine residues of this DNA. It was established (see Table 1) that the degree of methylation of cytosine residues in DNA of adrenalectomized rats 3.5 h after injection of cortisol does not change significantly as compared to DNA of rats of both control groups.

Therefore, rapid transcription response of cells to hormones is not associated with marked change in sensitivity of chromatin to DNase I or degree of methylation of DNA. This means that hormone-dependent loci of the genome in competent cells should originally possess "potentially active" conformation determined by increased sensitivity to DNase I and under-

methylation of individual DNA sites. These conclusions agree well with data obtained on analogous [19, 23] and similar systems [24-26] of hormonal induction of genome expression.

Apparently, at least two types of hormonal regulation of transcription should be differentiated. The first type is regulation of metabolism of cells already differentiated. Final response here begins rather rapidly, and methylation and sensitivity of induced loci of the genome to DNase does not change. A typical model of this type was investigated in the present work. The other type is differentiation of organs and tissues induced by hormones. This process occurs considerably more slowly and may be associated with cellular proliferation and such changes in specific sections of the genome which affect the level of both their methylation and their sensitivity to DNase I [27-29]. The latter data indicate that having once originated in the course of differentiation, potentially active conformation of hormone-dependent genes is very stable and is retained independently of whether functioning of the given gene continues or not [28, 29]. Apparently, "fast" and "slow" types of regulation may govern the so-called early and late effects of hormones [30].

CONCLUSIONS

1. Sensitivity of chromatin DNA of rat liver to DNase I and the level of its methylation do not change noticeably 3.5 h after administration of an inducing dose of hydrocortisone to animals.

2. Loci activated by glucocorticoids must initially possess potentially active conformation sensitive to DNase I.

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STATUS OF TYPE-II GLUCOCORTICOID RECEPTORS IN TISSUES OF ADRENALECTOMIZED RATS

P. P. Golikov* and N. Yu. Nikolaeva

UDC 612.014.47:577.175.53]-08

The mechanism of effect of natural and synthetic glucocorticoids is mediated by specific glucocorticoid cytoplasmic receptors of type II [1-3]. Only in complex with a specific receptor is a glucocorticoid hormone capable of activating the function of a cell's genetic apparatus and exerting an influence on intracellular metabolism [2]. Tissues with a high content of type-II glucocorticoid receptors possess greater sensitivity to the effect of glucocorticoids. Such tissues include, first of all, lymphoid and liver tissue [2]. In addition, type-II glucocorticoid receptors are also found in many other tissues [2-3].

The goal of the present investigation was a study of the status of glucocorticoid receptors of type II in different tissues of adrenalectomized animals.

MATERIALS AND METHOD

Investigations were conducted on male rats of the Wistar line (100-120 g) which were adrenalectomized 4 days before the experiment. Used in the work were triamcinolone 1,2,4-^{3}H-acetonide (T-AC) (22 Ci/mmole) from the Amersham company (England) and unlabeled T-AC from the Calbiochem company (USA). The status of type-II glucocorticoid receptors was determined in liver, kidneys, heart, and lungs according to Beato and Feigelson [2] with minor additions. Before extirpation of organs, the latter were washed through the lower vena cava with 50 ml chilled phosphate buffer (6.5 mM, pH 7.4) containing 150 mM sodium chloride. At all subsequent stages of the investigation, a strict temperature regime was observed (0-4°C). Tissue homogenization was performed in a glass homogenizer with a Teflon pestle at a rotation rate of 1000 rpm (two times for three passages with intervals of 30 sec). The ratio of tissue and buffer was 1:3. For homogenization of tissue, preparation of carbon suspension, and dilution of steroids, a buffer was used which contained 10 mM Tris-HCl, 1.5 mM EDTA, and 0.5 mM dithiothreitol, pH 7.4 [4]. Homogenate was centrifuged in a BAK-601 centrifuge (German Democratic Republic) on an angular rotor at 110,000g for 1 h. After centrifugation the upper layer of lipids was removed and supernatant was used to determine binding of ^{3}H-T-AC. Determination of binding of T-AC in tissue cytosol was conducted in test tubes for radiological investigation (LKB Company, Switzerland). Cytosol at 100 µl was introduced into each tube which contained 200-300 mg of protein [5], ^{3}H-T-AC in 100 µl of buffer in increasing concentrations from $0.25 \cdot 10^{-8}$ to $4 \cdot 10^{-8}$ M along two parallels to each point without addition of unlabeled steroid and to reveal nonspecific binding with the addition of a 100-fold excess of unlabeled T-AC in 100 µl of buffer (to complete the volume). Samples were mixed and incubated for 90 min at 0-2°C. After conclusion of incubations, free and bound ^{3}H-T-AC were separated by adsorption of free hormone by a suspension (100 µl) of A-quality activated carbon (3.75%) coated with dextran T-500. Carbon with free steroid was separated by centrifugation in a Hettich-Rotix centrifuge (LKB, Sweden) at 2-4°C for 10 min at 2000g. Supernatant at 200 µl was transferred into measuring flasks, 10 ml of ZhS-8 scintillation liquid was added, and it was counted on an Ultrabeta liquid scintillation counter (LKB, Switzerland). The number of binding sites for T-AC in cytosol of tissues and dissociation constants of the glucocorticoid receptor—T-AC complex were determined according to Scatchard [6]. Determination of protein in cytosol was made according to Lowry et al. [7].

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0097-0549/86/1604-0289$12.50