ΒΙΟΛΟΓΙΑ.— **The mechanism of steroid hormones action.** Ἀνεκοινώθη ὑπὸ τοῦ Ἀντιπροέδρου τῆς Σερβικῆς Ἀκαδημίας κ. Dušan Kanazir *.

INTRODUCTION

One of the crucial problems of today's molecular biology is the problem of hormonal regulation of the gene expression in the cells of higher organisms. Although during the past decade a considerable insight was gained into the first step of the hormonal regulation of genome expression in mammalian and human cells, we still do not know the basic mechanism (s) of hormonegenome interactions, that is the mechanism (s) of hormone regulatory protein interactions.

Up to now information on hormone action has been derived mostly from the study of mechanisms of the female sex hormones action.

On the basis of a variety of experimental evidence it appears that steroid hormones interact with target tissues by a two-step mechanism. In the first step hormone — the chemical signal — binds to a cytoplasmic receptor, which is presumably a characteristic component of each target cell. This hormone - receptor complex is translocated to the nucleus, where it associates with specific acceptors of chromosomes, again a characteristic component of the chromosomes of target tissue cells. This interaction accelerates the rate of transcription of the corresponding structural genes and activates the gene function. By this second step the cell machinery is switched on for a specific response.

In all these studies the gene activation by hormones was followed by a changed rate of activity of several enzymes. Thus, it was established that administration of corticosteroid hormones to rats results in an increased activity of several enzymes in liver cells.

In our laboratories of the Institute for Biological Research and Institute of Nuclear Sciences «Boris Kidrič» an extensive study of the cortisol (glucocorticoid) action on the genome of anabolic, catabolic tissues, as well as on non-target cells is in progress. In this study we are attempting to characterize the cortisol receptor and hormone - receptor complex, to bring more light into the hormone - receptor complex and

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* D. KANAZIR, **Μηχανικὴ ἐνέργεια τῶν στεροειδῶν ὁρμονῶν.**

chromatine interactions, as well as into regulatory mechanisms of the receptor - protein synthesis in target tissues.

In this communication I shall try to summarize briefly the results recently obtained by my colleagues: Drs. D. Trajković, N. Ribarac - Stepić, R. Metlaš and S. Popić, related to the mechanism of cortisol action.

PROPERTIES AND PURIFICATION OF HYDROCORTISONE RECEPTOR - PROTEIN (S)

The model systems in which we are studying the cortisol action are as follows: rat liver anabolic target organ, rat lymphocytes — catabolic tissue and cortisol resistant lymphocytes as non — target tissue.

I shall summarize the results related to the rat liver as anabolic target organ.

The hydrocortisone (cortisol) - receptor complex has been isolated from the cytoplasm of liver cells after administration of the labelled hormone (1, —2, —3_H - cortisol, specific activity 44CI/mMole; New England Nuclear Corporstion) or from the liver tissue cytosol, when the cytosol in vitro was exposed to tritiated (labelled) hormone. The complex has been purified by ammonium sulphate precipitation; sephadex - G - 25 filtration; DEAE - cellulose chromatography and agarose gel electrophoresis. The receptor appears to be mainly a specific protein, i. e. it is destroyed by proteases, and not by nucleases.

The biological activity of the cortisol - receptor complex has been studied in an «in vitro» system, following the action of the hormone - receptor complex on the rate of RNA synthesis in isolated liver nuclei, as well as on the DNA - dependent RNA polymerases activities. The results may be summarized as follows:

On ion exchange chromatography and isoelectric focussing the hydrocortisone - receptor complex of the liver cell cytosol is resolved into two components. They differ in molecular size and charge. These proteins had been eluted from DEAE - cellulose column chromatography at 0.04 M and 0.18 M concentrations of KCL (Fig. 1); they have two different isoelectric points, one at pH 4.85 - 5.00 and another at pH 5.85-6.10.

But only one fraction, i. e. the complex receptor - hormone eluted at 0.04 M KCl, was found to be able to enhance the rate of incorporation

of ^{32}p and other labelled precursors into the RNA of incubated rat liver nuclei; whereas the complex eluted with 0.18 M KCl was inefficient.

This finding might suggest that the receptor is a complex multimer consisting of several subunits. When the hormone binds to the receptor molecule from cytoplasm, it may induce an alosteric change of its complex, leading to its desaggregation. One of these subunits may be involved in the transfer of hormone to the nucleus, where complexing with a nuclear protein fraction results in the nuclear receptor - hormone complex, which interacts with specific acceptor sites on the chromatine. Meanwhile, the role of the second component which binds to hormone, but is inactive in the stimulation of RNA synthesis, still remains obscure. It might be a part of the «trapping» mechanism for the excessive amount (non - physiological) of hormone, or it may be involved in the regulation at the translation level, or some other level of the regulation of genome expression.

THE ROLE OF RECEPTOR

Is receptor simply a transport system?

Data on the receptor - hormone complex indicate as well that the receptor system is not simply a transport mechanism to deliver hormone to the nucleus of the target cell; it is more likely that the receptor protein may play a role in some key processes of the regulation of gene function. The function of hormone may be to promote transformation of the receptor into an active form, which can bind to acceptor sites on the chromatine. The receptor transformation seems to be a critical process in hydrocortisone (or steroids) actions, as indicated by our experiments - the complex receptor - cortisol (transformed complex) only, and not the receptor, enhances the RNA synthesis in isolated liver nuclei (Fig. 3). Nuclei isolated from liver cells show a significant increase in their ability to incorporate radioactive precursors ^{32}p into RNA in vitro, after being incubated with the cytoplasmic receptor - hormone complex, and not with the cytosol receptor fraction alone. The stimulation takes place only in conditions when the receptor is specifically transformed, i.e. when an active complex receptor - cortisol is formed.

From our data, as well as from data obtained in other laboratories, it is evident that the receptor may have a key role in the hormonal regulation of the gene expression. If it is the case, then one can raise several questions, as follows: is there a pool of receptor proteins in target cells, how the level of receptor protein in target tissues is regulated, does cortisol (hormone) stimulate and regulate the rate of synthesis of its own receptor, and at what moment after the hormone administration.

My colleagues are trying to get some answers to these questions. Therefore they started a study of the rate of early protein synthesis within the first 60 minutes after the hormone administration.

I shall briefly summarize our findings derived from various experimental approaches.

1. Within the time interval between 10-60 minutes there is no observed difference in the rate of labelling a bulk of cytoplasmic-soluble proteins by comparing the cytosols of controls and of the hormone treated animals.

2. But using methods of labelling the complex and methods of its purification it became evident that cortisol enhances the synthesis of cortisol specific receptor proteins in the rat liver. There are two peaks of receptor synthesis: 10 and 60 minutes after the hormone administration.

3. Ten minutes after the hormone treatment cytosol was separated on agarose gel electrophoresis and the fraction migrating 5.5 cm from the origin was most labelled. It was found to bind to the maximal amount of hormone. That is the only fraction which seems to be synthesized very early after the hormone administration.

The pattern of labelling liver soluble proteins after electrophoretic separation on agarose gel electrophoresis suggests that hydrocortisone induces the synthesis of specific receptor protein at a very early time interval, 10 minutes after hydrocortisone administration. This finding represents an evidence that hydrocortisone stimulates in the liver very early «de novo» synthesis of its own receptor.

Pre-treatment of animals with puromycine (2 mg/100 gr) 10 minutes before injecting hydrocortisone and ^{14}C-amino acids abolishes almost completely the early synthesis of receptor protein, detectable 10 and 60 minutes after the hormone administration, as judged by agarose gel electrophoresis and ammonium sulphate precipitation. The early synthe-

sis of the specific receptor is also inhibited by pre-treatment of animals with actinomycine D (250 mg/100 gr) 30 minutes before hydrocortisone administration.

These data suggest that only the early protein, the synthesis of which was induced by hormone, is the specific receptor for cortisol.

Is the early protein a specific receptor?

To test the biological activity of the newly synthesized receptor-hormone complex we used isolated nuclei to study the activity of RNA polymerases. The hormone-receptor complex, formed in vivo or in vitro, purified by gel filtration or agarose gel electrophoresis, was incubated with isolated liver nuclei. The activity of RNA polymerase A (I or low-salt), characterized by greater activity at low ionic strength, and RNA polymerase B (II or high-salt), localized in nucleoplasm and characterized by increased activity at high ionic strength, have been tested.

The activity of RNA polymerase B shows a significant increase within the first 10 minutes of incubation. There is no alteration of RNA polymerase A activity during this short interval. But the polymerase A reaches a peak at 30 minutes, when the activity of polymerase B begins to decrease. Within the first 60 minutes of isolated nuclei incubation with the cytoplasmic complex the activation of both RNA polymerases in the nuclei was observed. These findings well agree with the results related to total nuclear RNA synthesis (shown in Fig. 6) in vivo after hydrocortisone administration. The second rise in the activity of polymerases in isolated nuclei was observed after 2 hours of incubation. The peak of the restimulated activity was observed at 3 hours of incubation; it was then followed by a gradual fall until 18 hours. The RNA polymerase A activity continued to rise from the 3rd hour, with a secondary rise to a peak at 6 hours.

Thus, the activation of nuclear RNA polymerase within the first 30 minutes may be responsible for the synthesis of receptor protein in cytoplasm which binds to cortisone.

Is the cytoplasmic receptor-hormone complex itself an active complex regulating the gene expression?

In an attempt to answer this question we treated purified nuclei prepared from the liver, thymus, kidney, spleen and cortisol resistant lymphocytes with homologous and heterologous cytoplasmic ^{3}H cortisol-receptor complexes. Incubation of these mixtures was done at $37^{0}C$ for 15 minutes. Following the time interval of incubation nuclear proteins soluble in 0.3 M NaCl, namely non-chromatin proteins, were extracted from incubated nuclei and submitted to get filtration on sephadex 6-75 column in order to separate ^{3}H cortisol-nuclear protein complexes from free radioactivity, i. e. released hormone.

When the liver cytoplasmic receptor-^{3}H cortisol complex, isolated from the liver, of cortisol-treated animals, was incubated with the liver, thymus, kidney, spleen and lymph nodes nuclei of untreated animals, the bound radioactivity, i. e. nuclear proteins-hormone complexes were found in the nuclei of the liver thymus and kidney cells, but not in the nuclei of the spleen and lymph nodes.

However, when the mixtures of the liver nuclei (prepared from the liver of untreated animals) were incubated with cytoplasmic hormone-receptor complexes, prepared from the thymus, kidney, spleen and lymph nodes, nuclear protein-cortisol complexes were found in all samples. The specific radioactivity of nuclear complexes has always been significantly higher in mixtures with liver nuclei. In all these mixtures of nuclei, where nuclear protein-hormone complexes were found, the rate of RNA synthesis was enhanced, i. e. the genes were activated.

In addition it should be mentioned that in vivo conditions the cytoplasmic complex was found in all mentioned tissues: the liver, thymus, kidney, spleen and cortisol resistant lymphocytes, whereas nuclear complexes were found only in the liver, thymus and kidney cell nuclei.

From the foregoing observation it is evident that the responsiveness of target tissue cells to cortisol depends on more than the presence of cytoplasmic cortisol-receptor proteins. Therefore the formation of autoplasmic receptor-cortisol complex does not necessarily mean the activation of genome; some other nuclear protein components seem to

be necessary for the formation of an active hormone - receptor complex, which is able to recognize acceptor sites on the chromosomes of target cells, i. e. which stimulates the activation of genes.

How does the complex act upon the constituents of the chromatine-genome?

In our studies of the mechanism of hormone action we were interested in cortisol - histones and cortisol - acidic proteins interactions. Both classes of proteins represent the main constituents of chromosomes.

For that purpose the nuclei of liver cells were incubated with the complex and ^{32}p. The complex enhances the ^{32}p's incorporation into the rat liver nuclei, as well as the rate of RNA synthesis. The rate of phosphorylation of histones was also markedly increased. It was 75% higher than the rate of phosphorylation in the control samples of nuclei, i. e. nuclei + cytosol (non - transformed receptor protein). As it has been proposed, the induction of RNA synthesis might be brought about by a change in DNA - histone interaction, resulting from histone phosphorylation; this would presumably involve a change in configuration of histones with a consequent derepression of DNA - genes, followed by RNA and protein synthesis.

Non - histone chromosomal proteins, especially phosphoproteins, have aroused considerable interest as possible regulators of the gene activity. We studied the effect of cortisol on phosphorylation of phosphoproteins and their role in the nuclear RNA synthesis, i. e. in the genes activation. Cortisol stimulates the incorporation of ^{32}p into phosphoproteins of the rat liver nuclei 20 minutes after the hormone injection. The addition of the phosphorylated phosphoprotein fraction to the liver nuclei stimulates the biosynthesis of nuclear RNA.

Our results seem to be compatible with the postulated regulatory role of phosphoproteins in gene expression.

CONCLUSIONS

The cortisol binding receptor from the rat liver can be resolved in two components of which only one is transformed into an active complex that recognizes receptor sites of chromosomes and brings about genes

activation; the second of them seems to have the hormone-trapping function.

The receptor alone has no ability of stimulating the gene activity, i.e. transcription. Cytoplasmic cortisol receptors have been found even in non-target cells. Therefore the formation of the cytoplasmic receptor-cortisol complex does not necessarily mean the initiation of genome activation; some other component-specific nuclear protein is needed to transform in nuclei the cytoplasmic complex into a complex able to activate genes function, i.e. to recognize acceptor sites on the chromosomes of target cells. In this transformation a non-chromatin protein seems to be involved.

The nuclear complex has been found only in the nuclei of target cells. It sequentially activates both DNA-dependent RNA polymerases; nuclear polymerases, transcribing structural genes, seem to be activated first, within 10-30 minutes time interval, following the cortisol administration. Sixty minutes after the hormone administration nuclear RNA polymerases — transcribing ribosomal genes — are activated. The only protein synthesized in the liver very early after the cortisol administration, within 10-30 minutes time interval, is the specific cortisol receptor. Therefore it seems reasonable to assume that the «pool» of cortisol specific receptor-proteins is regulated by cortisol itself, i.e. by its amount reaching the cell. This synthesis was prevented by actinomycine D and puromycine. It can accordingly be assumed that «de novo» synthesis of receptor proteins takes place after the cortisol administration.

The receptor-cortisol complex stimulates chemical modifications of histones and phosphoproteins of chromosomes. In the presence of complex the phosphorylation rate of both chromosomal constituents is increased in the nuclei. The structural modification of these proteins seems to be a prerequisite of the enhanced transcription of structural genes.

On the basis of data presented, as well as on the basis of other experimental evidence, obtained in our laboratories, we propose a modification of the present «two-step» overall picture of the mechanism(s) of steroid hormone action in target cells. Up to now the two-step sequence: cytoplasmic receptor-hormone formation and its nuclear translocation has been accepted to present a general pattern of steroid hormone action. We believe that steroid hormones act by a modified «two-

step» pattern. Namely, the formation of cytoplasmic receptor - hormone complex does not necessarily mean that the «two-step» sequence action is initiated. This view is supported by the fact that cytoplasmic proteins, binding steroids, were found even in non-target tissues.

Only after translocation of this complex to the nuclei it seems to be transformed into a regulatory complex, by associating with a nuclear protein, which is a specific constituent of target cell nuclei. After such transformation the complex was enabled to recognize acceptor sites on the chromosomes and to stimulate the transcription, i. e. gene activation.

CONCLUSIONS

On the basis of a variety of experimental evidence, obtained in our laboratories, it appears that cortisol interacts with target tissues by a two-step mechanism in which the hormone - chemical signal - binds to a cytoplasmic receptor, which is a characteristic component of target cell (liver cells). The cortisol-receptor complex is translocated to the nucleus, where it associates with specific acceptors of chromatin, again characteristic of target tissue and in some unknown way it accelerates the rates of transcription of structural and ribosomal RNA genes.

The cortisol binding receptor seems to be multimer - specific protein, consisting of several subunits. Only one of these subunits is transformed in such a way to enhance the rate of transcription. The receptor alone has no ability to stimulate the rate of transcription in isolated liver nuclei. Therefore the key event in initiation of the response of target cells to hormone seems to be the transformation of cytoplasmic receptor due to binding to the hormone C/2.

The relation between the cytosol and nuclear cortisol - receptor complex enhances the rate of RNA polymerases activity in vivo, as well as in vitro, that is in isolated liver nuclei, but this effect was not observed in the nuclei isolated from nontarget organs.

The specific cortisol receptor was detected among the early protein «de novo» synthesized 10 to 60 minutes after the administration of hormone. This synthesis was preceded by nuclear RNA synthesis and was prevented by actinomycin and puromycin.

The two-step sequence involving nuclear translocation of a cytoplasmic receptor-hormone complex appears to represent a general pattern of steroid hormone action.