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CORTISOL DEPENDENT ACUTE METABOLIC RESPONSES IN RAT LIVER CELLS

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SUMMARY

Cortisol injected into the rat induces immediate metabolic changes in liver cells, occuring within time intervals shorter than that accounted for by the expression of specific genes. Among these extragenomic acute metabolic responses we describe: the enhanced rate of the synthesis of receptor protein for cortisol, the increase in overall capacity for protein synthesis and increased rate of phosphorylation of histones and nonhistone proteins. All these changes depend on cortisol and occur within the first 30 minutes after hormone administration. All these acute metabolic responses to cortisol might be correlated to the hormone-produced activation of the native macromolecular receptor-system. In an attempt to integrate extragenomic and genetic responses to cortisol a new concept and hypothesis of cortisol action is proposed, which can probably be extended to other steroids and extrapolated to other steroid responsive tissues.

INTRODUCTION

One of the crucial problems of today's molecular biology is the problem of hormonal regulation of the gene expression in eucariotic target cells. Although a considerable insight into the role of steroid hormones in physiological regulation was gained during the past decade, the basic mechanism(s) of hormone–genome interactions in target cells, i.e. molecular mechanisms of the selective regulation of the expression of a relatively small number of specific genes in target cells, as well as molecular mechanisms underlying acute responses to steroids still remain obscure.

A large body of experimental *in vivo* and *in vitro* data is consistent with the idea that various steroid hormones function via a common two-step molecular mechanism. In the first step the steroid hormone—allosteric effector—binds tightly to its specific cytoplasmic receptor molecule, which is presummably a characteristic component of each target cell. The binding event induces activation—alosteric transition—of the steroid receptor. The complex hormone–receptor, which has an increased affinity for chromosomal acceptor sites, is translocated to the target cell's nucleus and interacts with chromosomal acceptor sites. This steroid–receptor complex interaction with the genome triggers and selectively accelerates the rate of transcription of a few structural genes. By this second step the cell machinery is switched on for specific biological responses, characteristic of the particular steroid hormone and target tissue[1–4].

According to that model the steroid receptor seems to play a key role in the control of gene expression in eucariotic target cells. An extensive study on cortisol specific receptor[4, 5] and its regulatory role[6, 7] in genetic expression, which is in progress in our laboratory, lead us to the working hypothesis which will be presented in this paper.

We propose that the native cytoplasmic steroid receptor represents a macromolecular system, consisting of several regulatory proteins—subunits. This receptor system appears to comprise a "code" for multiple cooperative metabolic functions, which seem to proceed simultaneously after the hormone induced activation of the receptor-system. By binding to the specific receptor the hormone produces disaggregation and conformational transition of the receptor-system. In the proposed concept the activation of receptor is a crucial event, which may trigger a series of immediate effects, seen in target cells, with time intervals shorter that those accounted for by the hormone-induced gene expression.

Among the acute effects of cortisol in the rat liver, we observed: early synthesis of cortisol specific receptor, phosphorylation of histones and non-histone proteins and the early overall activation of translatory machinery. The inhibitors of RNA-synthesis did not affect these immediate processes. They occur within time intervals markedly shorter than those accounted for by gene expressions, i.e. hormone induced *de novo* synthesis of some specific proteins.

In our view these and other points are sufficient to question the validity of our present concepts of the role of the steroid receptor. At the present moment the receptor function was only attributed to that subunit of receptor which specifically binds the steroid hormone (steroidophilic subunit). Having in mind the integral structure and integrated functions of the steroid receptor, we propose that the activation of the receptor controls the rate of modifications of existing proteins, as well as the expression of specific genes. It seems that some events, occurring in the target cells very soon after hormone administration,

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are necessary for the selective expression of a few specific genes in the target cells.

In our studies we attempted to answer several questions relevant to the structure and function of cortisol receptor such as: how the level of native receptor molecules—its pool size—is regulated in the target cells; how does the "activation" of receptors act upon the constituents of the chromatin-genome of target cells; is the translation machinery stimulated at the moment of receptor activation, i.e., before the newly synthesized RNA has been mobilized into active polysomes, after hormone administration; what are the molecular mechanisms underlying the acute responses to cortisol etc.

In this paper we will present some of our recent data which favor the support of our hypothesis.

EXPERIMENTAL

Animals. Male Mill Hill hooded rats, weighing 200–250 g were used. The animals were fed and watered *ad libitum.*

Materials. [1,2-^{3}H]-cortisol (44 Ci/mmol), ^{14}C labelled amino acids (1 Ci/ml), ^{3}H labelled amino acids (1 Ci/ml), [^{3}H]-uridine (3 Ci/mmol) and [^{3}H]-UTP (50 Ci/mg) were purchased from New England Nuclear Corporation. Agarose was a product of Milles-Servac.

Buffers. In most of the experiments 0.25 M sucrose, 0.025 M KCl, 0.01 M $MgCl_2$ and 0.05 M Tris–HCl, pH 7.55 was used. Electrophoresis was performed in barbital–sodium barbital buffer, 0.075 M, pH 8.6, containing 2 mM calcium lactate.

Injection of hormone. Labelled and unlabelled hormones were injected, using a dose of 50 μCi, or 2 mg/100 gm body weight. Controls received injections of 0.14 M NaCl. The animals were killed at various time intervals, after hormone injection, and their livers were immediately perfused *in situ*, through the portal vein, with cold buffer.

Measurement of the rate of protein synthesis in vivo. Rats were injected intraperitoneally with ^{3}H labelled amino acids, using a dose of 50 μCi/100 gm body weight (controls) and ^{14}C labeled amino acids (the same dose) and cortisol (treated). The duration of this treatment is stated in the text. In certain experiments with actinomycin D the inhibitor was dissolved in 0.9% NaCl adjusted to 2% ethanol. Animals received intraperitoneally 250 μg/100 g body weight of actinomycin D 30 min before treatment with hormone or labelled precursors.

Preparation of cytosol and nuclei. The cytosol fraction was prepared exactly in the same way as described by Beato *et al.*[8]. In all experiments the nuclei were isolated in 2.2 M sucrose, by the method described by Chaveau *et al.*[9]. In order to obtain partially purified cortisol–receptor complex (in certain experiments), cytosol fractions were subjected to gel filtration on column of Sephadex-G-25, or the excess of labelled steroid was removed by charcoal treatment (10% w/v charcoal, 1% w/v dextran). The fractions comprising proteins with a bound hormone were salted out with the increasing concentration of ammonium sulphate. The cortisol binding protein (receptor) precipitated in the concentration between 20–35%[5].

Agarose gel electrophoresis. This method has been described in detail elsewhere by Johansson[10]. Agarose gels (1%) were made in barbital–sodium barbital buffer, pH 8.6. A suspension of agarose is heated and spread on a 11 × 20 × 0.1 cm glass plate in a strictly horizontal position, using the slit forming device for preparation of regularly formed slits in the gel. The samples of proteins (10 μl) were placed in the slits and electrophoretic separation was run with a potential gradient of 20 V/cm. Electrophoresis was performed in barbital–sodium barbital buffer (pH 8.6) for 50 min. The gel temperature was maintained at 5°C. For determination of proteins and measurement of radioactivity the strips from four or five plates were pooled (sectioned into 2 mm wide fractions) and homogenized in TSS buffer. The gel suspensions were centrifuged for 10 min at 3500 rev./min.

RNA polymerase assay. The complex between cortisol and receptor protein (formed *in vivo*, or *in vitro*) was tested in the system of purified nuclei for their effects on the rate of biosynthesis of RNA. The assay for endogenous RNA polymerase activity in isolated nuclei was described by Roeder and Rutter[11] and Glasser *et al.*[12]. Incubations were carried out at 25°C and terminated by addition of cold 10% TCA. The pellets were collected on filters (Millipore filters, 0.45 mμ pore size) and DNA was solubilized by hydrolisis in 0.3 M $HClO_4$. The specific radioactivity (c.p.m./mg DNA) was calculated from the c.p.m./filter and the μg DNA on the same filter.

Preparation of nuclear RNA. The nuclear RNA's were prepared from purified nuclei according to the procedure of Maramatsu *et al.*[13].

Determination of protein and DNA. Protein was determined by the method of Lowry *et al.*[14] with bovine serum albumin as a standard. DNA was determined by the method of Burton[15] with calf thymus DNA as a standard.

RESULTS

I. *Cortisol dependent immediate modifications and stimulation of translational machinery in rat liver cells*

The main concept of our working hypothesis postulates the regulatory action of the subunits of the receptor-system after its activation, i.e. dissociation of subunits following the binding of specific steroid. We also postulate that the targets of their actions might be translational machinery and membrane systems of the cells responding to steroids. To probe this concept we explored the immediate effects of injected cortisol on the translational capactity of target cells.

It is well established that a single injection of cortisol in the rat liver cells increases the rate of induced

enzyme synthesis within time intervals ranging from 4–12 h after cortisol injection[16–18]. These data suggest that the selective expression of a series of structural genes seems to proceed slowly, compared with the acute responses observed in the first hour after hormone administration. In an attempt to explore these acute responses of rat liver cells to cortisol we studied *in vivo* and *in vitro* conditions of the protein synthetizing capacity of rat liver cells.

For that purpose we followed *in vivo* rate of incorporation of the labelled amino acids into cytosol proteins of rat liver cells within 120 m after cortisol administration.

1. *Rate of incorporation of ^{14}C-labelled amino acids into liver cytosol proteins.* The time-course of the incorporation of ^{14}C-labelled amino acids into liver cytosol proteins was followed in order to investigate whether or not selective early synthesis of proteins takes place under the influence of cortisol.

Within the 120-min time interval two peaks of protein synthesis were detected, 10 and 60 min after cortisol and labelled amino acids administration. When these fractions were identified it appeared that they belonged to the receptor for cortisol.

Figure 1 illustrates the synthesis of cortisol receptor protein, partially purified by ammonium sulphate precipitation in the concentration between 20–35% of saturation.

The maximum rate of incorporation of amino acids into proteins was reached 10 and 60 min after cortisol administration, representing values 130% and 201% of those obtained in the corresponding controls.

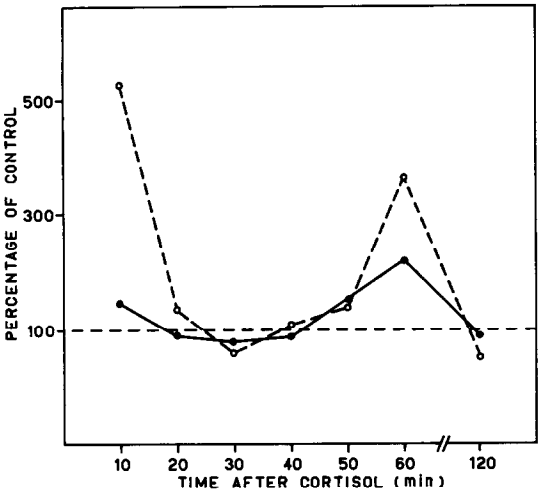

Fig. 1. Time-course of the induction of synthesis of cortisol-receptor protein. The animals were injected with cortisol and ^{14}C-labelled amino acids and sacrificed after 10, 20, 30, 40, 50, 60 and 120 min after hormone treatment. The cytosol fractions were precipitated by 20–35% saturation of ammonium sulphate. The precipitates were collected, resuspended in buffer and used for determination of proteins and radioactivity (●——●). Cytosol fractions were prepared at indicated times and submitted to agarose gel electrophoresis and receptor protein for cortisol was eluated as described under Materials and Methods (O-----O).

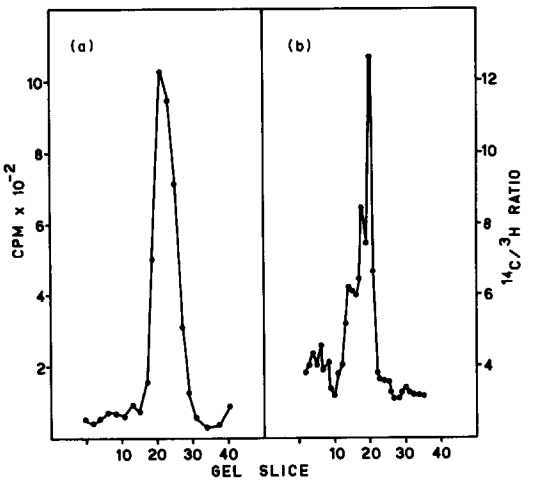

Fig. 2. Electrophoretic separation of cortisol-receptor protein. The cytosols prepared from rat livers were incubated with [^{3}H]-cortisol (30 min at 25°C), were treated with charcoal-dextran suspension 10 min at 0°C and were precipitated by ammonium sulphate. Redissolved precipitates were subjected to electrophoresis (a). Cortisol treated animals were labelled with ^{14}C-labelled amino acids and controls were labelled with [^{3}H]-amino acids (b). Electrophoresis: 10 µl samples run in 1% agarose gel in 0.075 M barbital–sodium-barbital buffer, pH 8.6. The gel was sliced and radioactivity in the slices was determined.

The results shown in Fig. 1, also indicate an increase in the incorporation of label into the band corresponding to the receptor protein after electrophoretic separation of total cytosol proteins.

Within the first 120 min after cortisol administration no increase of labelling in total rat liver soluble proteins was observed. During this time two peaks of cortisol specific receptor synthesis were detected. The increase in the rate of incorporation of the labelled amino acids into receptor molecules was four and five time higher 10 and 60 min after cortisol administration than it was in the controls.

The rate of receptor synthesis was also measured by isotope ratio technique.

Figure 2 shows the electrophoretic separation of the cortisol–receptor complex prepared by ammonium sulphate precipitation and ^{14}C/^{3}H ratios of the receptor protein.

The rat liver cytosols were incubated with final concentrations of 10^{-18} M labelled cortisol and after charcoal treatment were precipitated with 20–35% ammonium sulphate. The precipitate was resuspended in buffer and an aliquot was subjected to electrophoresis (Fig. 2a). When cortisol binding was preformed on the charcoal treated cytosol no detectable radiactive steroid, associated with other protein fractions (after gel electrophoresis) was found, which would suggest a high affinity of the newly synthesized receptor for cortisol.

Figure 2b illustrates ^{14}C/^{3}H ratios from a double-isotope analysis, in which rats were injected with cortisol and ^{14}C-labelled amino acids for 60 min, while untreated control animals were treated with ^{3}H-labelled amino acids. The cytosol fractions from

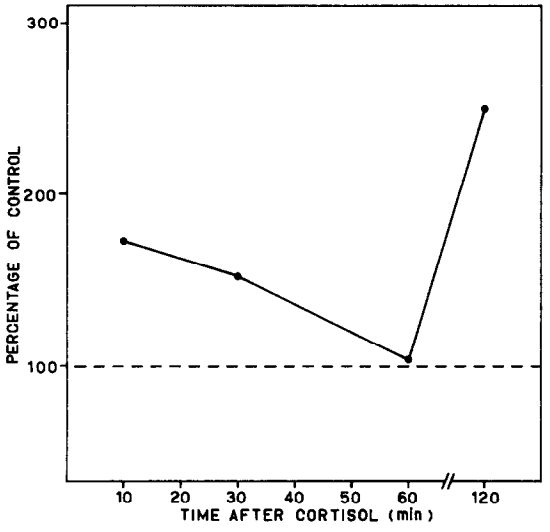

Fig. 3. Effect of cortisol upon the synthesis of total ribonucleic acids in nuclei. The animals were injected with [^{3}H]-uridine (100 μCi/animal) and cortisol and killed at indicated times. The total ribonucleic acids were extracted from purified liver nuclei, using SDS-phenol solution at the temperature of 60°C.

livers were pooled and subjected to ammonium sulphate precipitation and electrophoresis in agarose. The receptor synthesis is indicated in Fig. 2b by the increase in $^{14}C/^3H$ ratio, corresponds to the bands which bind ^{3}H-cortisol (Fig. 2a).

2. *Changes in the rate of [^{3}H]-uridine incorporation into nuclear RNA after cortisol treatment.* An attempt has been made to correlate the early synthesis of cortisol receptors to the labelling rate of nuclear RNA.

Figure 3 shows the time-course of the incorporation of [^{3}H]-uridine into RNA after a simultaneous injection of labelled uridine with cortisol. As shown in Fig. 3, this study confirms the hormonal enhancement of hepatic RNA synthesis. The rate of uridine incorporation into RNA increases during the first 30 min after cortisol administration. The second rise of uridine incorporation was observed 120 min after hormone administration.

The increased rate of RNA synthesis within a 30-min interval after hormone administration has the same time-course with the first peak of receptor protein synthesis and precedes the second peak.

3. *The effect of the receptor protein–cortisol complex on the liver RNA polymerase activity.* In order to test the biological activity of the newly synthesized receptor, isolated rat liver nuclei were incubated with complex cortisol–receptor and the activity of RNA-polymerases was followed.

The response of liver nuclear RNA polymerases after incubation of isolated liver nuclei with the receptor protein–cortisol complex is shown in Fig. 4.

Cortisol–receptor complexes prepared with newly synthesized receptor from subcellular fractions of the rat liver have significant stimulatory effects on the activities of RNA polymerases of rat liver nuclei.

As can be seen (Fig. 4) the high-salt polymerase (B) activity showed a significant increase within the first 10 min, when the rat liver nuclei were incubated with this complex. There is no change in low-salt polymerase activity during this short time interval of incubation. The activity of polymerase A begins to rise later, after 30 min of incubation, at which time the activity of polymerase B begins to fall.

These findings are in a good agreement with the results related to total nuclear RNA synthesis *in vivo* (shown in Fig. 3) after cortisol administration and [^{3}H]-uridine.

The response of nuclear polymerases to that complex seems to prove that the newly synthesised protein fraction is a receptor protein for cortisol. This finding indicates that cortisol controls the rate of synthesis of its specific receptor.

4. *Does actinomycin D affect the early synthesis of receptor protein?* The effects of actinomycin D on cortisol dependent stimulation of receptor and RNA synthesis in the liver cells were also examined *in vivo*.

Pretreatment of animals with actinomycin D 30 min before injection of cortisol and ^{14}C-amino acids did not affect 10 min peak of receptor synthesis but almost completely abolished the synthesis of receptor protein, which was observed 60 min after hormone administration (Fig. 5).

RNA synthesis stimulated by cortisol for the first 30 min has also been inhibited after actinomycin D treatment. The data presented suggest that the appearence of new receptor protein 60 min after cortisol administration requires a preceding stimulation of RNA synthesis.

Our data obtained under *in vivo* experimental conditions indicate that an early, 10-min peak of receptor synthesis, which is unaffected by actinomycin D, can be attributed to acute responses of the rat liver cells

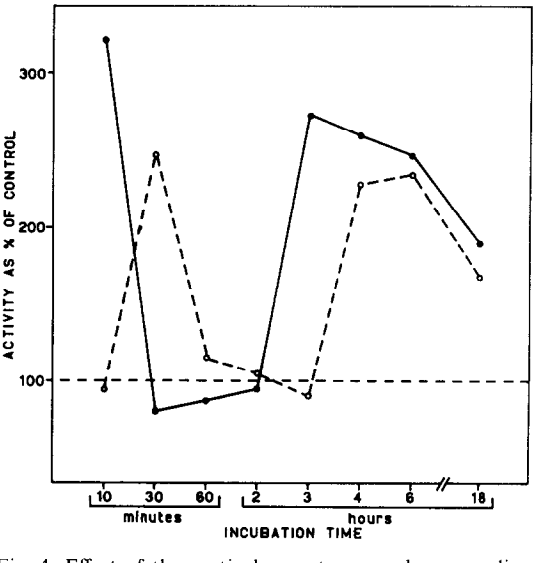

Fig. 4. Effect of the cortical–receptor complex upon liver RNA polymerases activity in isolated liver nuclei. Endogenous RNA polymerase activity in liver nuclei was assayed under high-salt conditions (●——●), or low-salt conditions (O-----O).

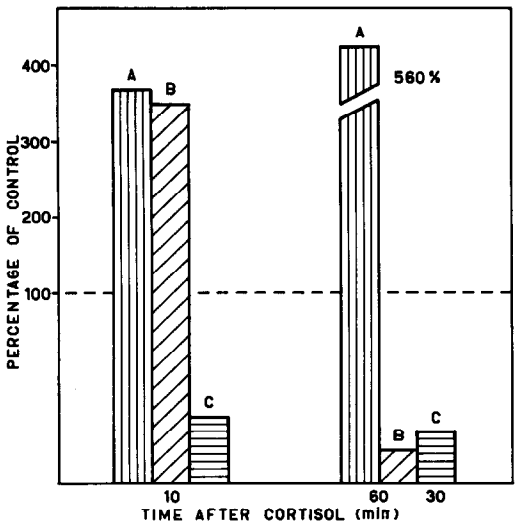

Fig. 5. Effect of actinomycin D upon the synthesis of cortisol receptor protein and nuclear RNA. (A) rats were injected simultaneously with cortisol and ^{14}C amino acids, 10 and 60 min. Receptor–cortisol complex was prepared by agarose gel electrophoresis. (B) rats were pretreated by actinomycin D (30 min) and injected simultaneously with cortisol and ^{14}C amino acids, 10 and 60 min. Receptor cortisol complex was prepared by agarose gel electrophoresis. (C) rats were preteated by actinomycin D (30 min) and injected simultaneously with cortisol and [^{3}H]-uridine, 10 and 30 min.

to cortisol since it occurs within shorter time intervals than that reported for the selective expression of specific structural genes.

This finding very strongly suggests that the activation of receptor may be the event which is involved in the selective translation of performed mRNA coding cortisol specific receptor.

II. *Stimulation of rat liver polyribosomes as an acute response to cortisol treatment*

Evaluation of various responses to steroids indicates that events other than specific gene expression steroid-dependent ones occur in the target cells. We postulated that the molecular mechanism(s) underlying these extragenomic events are dependent on hormone produced activation, i.e. on the disaggregation of the receptor-system and we assume that its subunits might be involved in the post-transcriptional and translational control. In an attempt to examine these postulates we are studying the immediate effects of injected cortisol upon the rate of nuclear RNA processing, and its transport to the cytoplasm, as well as upon the rate of translation.

Although research is in progress, our preliminary results already indicate an enhancement of polyadenylisation of nuclear transcripts within 30 min of cortisol injection, suggesting that the processing of nuclear transcripts is enhanced in some still unknown ways[19, 20]. Newly synthesized cortisol induced RNA, rich in poly-A, was transferred to the cytoplasmic pool of mRNA within 30–60 min after cortisol administration.

However, it is not yet clear whether the steroid mediated stimulation of RNA synthesis, i.e. the transcription in differentiated cells is a primary or secondary response. The primary response should be a rapid one, within the first hour after hormone administra-

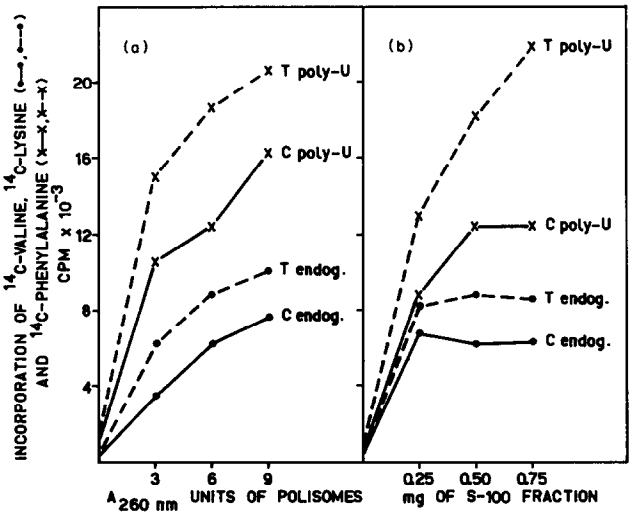

Fig. 6. Effect of cortisol *in vivo* on incorporation of amino acids *in vitro*. Animals were sacrificed 10 min after cortisol injection. Reaction mixture contained in volume of 100 μl: various amount of polysomes and S-100 fraction as indicated in Fig. 50 mM Tris, pH 7.6, 80 mM HM_4Cl, 6 mM Mg-acetate, 6 mM mercaptoethanol, 2.5 mM ATP, 0.5 mM GTP, 10 mM PEP, 10 μg PEPkinase, a mixture of 18 amino acids lacking valine and lysine (25 mM each) and 1.0 μCi of labelled amino acids for endogenous incorporation. The polyphenylalanine incorporation directed by poly-U was measured in the same system omitting the non labelled amino acids. 40 μg of poly-U was added per incubation mixture. Samples were incubated at 37°C for 30 min. The radioactivity of heat stable acid precipitable material was determined. (a) incorporation with different polysomes concentration. (b) Different S-100 concentrations. ●——●—endogenous incorporation of valine and lysine in controls. ●---●—in cortisol treated animals. × —— × —poly-U incorporated in control. × --- × —cortisol treated animals.

tion, that is before the translation of newly transcribed mRNA's, induced with hormone. Thus, for example, the induction of ovoalbumin mRNA is observed *in vivo*, in tissues other than oviduct, only after a 3 h lag, following estradiol administration[21, 22], whereas the increase of *in vitro* activities of RNA polymerases A and B of the rabbit uterus was observed 0.5–4 h after estradiol injection[23].

In an attempt to approach the problem of extragenomic events dependent on steroids, in our system on cortisol, we studied the capacity of protein synthesis of rat liver polyribosomes 10 min after administration of cortisol. Within this time interval there was no transfer of newly synthesized mRNA from the nuclei into the cytoplasm.

For this reason the animals were sacrificed 10 min after cortisol injection. The liver polysomes and S-100 fractions were prepared from both the treated and control animals, and in an *in vitro* system the incorporation of [^{14}C]-valine and [^{14}C]-lysine in polysomes has been followed. The phenylalanine incorporation, directed by poly-U in polyphenylalanine, was followed in the same system. Other amino acids were omitted and 40 μg of poly-U were added per incubation mixture. The samples were incubated at 37 C for 30 min. The radioactivity of heat stable acid precipitable material was determined.

The capacity of polyribosomes and the S-100 fraction to incorporate the labelled amino acids into proteins was found to be enhanced 10 min after cortisol administration *in vivo*.

Polysomes and S-100 fraction from treated animals showed, 10 min after cortisol administration, an increased endogenous mRNA directed synthetic protein activity. In the same time interval an increased capacity of rat liver ribosomes and S-100 fraction to translate poly-U was observed (Fig. 6a and b). Cortisol induced enhancement of poly-U translation suggests the modification of ribosomal proteins and/or specific factors involved in protein synthesis, such as initiation and elongation factors.

Since initiation factors are not obligatory components of *in vitro* translation of poly-U, our results suggest that the enhancement of translation capacity may be attributed to the modification of ribosomal proteins and to elongation factors.

Accordingly, we propose to take "receptor" as a protein modifying system. It might activate some protein kinase, held in a restricted, inactive state in the membrane structure until the receptor-system has been activated by steroids. This idea is strongly supported by the observation that an early increase in cAMP occurs as early as the detectable binding of estrogen to cytosol receptor protein[1].

III. *Gross effects on chromatin, induced by steroid receptor activation*

We attempted to examine the structural changes of chromatin by following the rate of chemical modifications of histones, as well as the modifications, i.e. phosphorylation of nonhistone proteins of chromatin. Our results show that the phosphorylation of histones as well as that of soluble cytosol proteins and nonhistone proteins is enhanced within 20 min of cortisol injection (Table 1). These phosphorylations are not inhibited by actinomycin D and cycloheximide, i.e. they are not dependent on hormone induced synthesis of proteins[4, 6, 7, 24]. Our data are in good agreement with data obtained in other laboratories.

Table 1. Effect of cortisol upon the phosphorylation of rat liver nuclear proteins

Protein fractions	Specific radioactivity	
	Controls	Cortisol treated
0.14 M NaCl	8.6	10.3
0.25 M HCl	3.0	4.1
Phosphoproteins	14.3	67.4

Results are expressed as counts per min $\times$ mg^{-1} $\times$ 10^3; they represent the average values of three experiments (reproduced from Ref. 7).

Thus, a transient increase in histone acetylation 5 to 10 min was observed after *in vivo* estradiol action on lymphocytes[25], and on uterus[26]; aldosterone action on kidney[25]. The fact that the effects disappear 20 min after hormone administration suggests a very fast turnover of acetate and phosphate groups and a fast chemical modification of histones.

It is also shown that insulin stimulates the synthesis of histones in mammary epithelial cells[27] and that gonadotropin accelerates the incorporation of [^{3}H]-lysine into histones 10 min after a single injection of hormone[28].

Increased rates of ^{32}P and ^{14}C-labelling of most nuclear proteins are seen at various time intervals following gonadotropin injection[28].

The nonhistone chromosomal proteins seem to be involved in the hormonal regulation of gene expression in target cells[2, 29, 30]. The rate of their phosphorylation is subject to hormonal regulation[31, 32].

The observation that this rapid rise in histone phosphorylation[24] and acetylation is unaffected by either actinomycin D or cycloheximide pretreatments[24–26] is consistent with the idea of our hypothesis, i.e. the involvement of chemical modifications of preformed proteins in the early hormone response, mediated by the metabolic code of activated receptor.

It is difficult to reconcile the high stimulation of histone and nonhistone protein modifications described above, with the activation of a limited number of specific genes in target cells. The biological meaning of these phenomena is still obscure.

DISCUSSION

The results presented in this paper demonstrate that cortisol treatment of the rats results in an immediate enhancement of the synthesis of specific proteins. Among newly synthesized proteins we identified

a cortisol specific receptor. The enhanced incorporation of amino acids into the receptor protein fraction revealed two peaks of receptor protein synthesis, 10 and 60 min after cortisol administration to the animals (Fig. 1). Within the same time interval no labelling of bulk soluble proteins of rat liver cytosol was observed. The pretreatment of rats with actinomycin D almost completely abolishes the 60-min peak of receptor protein synthesis (Fig. 5). The immediate synthesis of receptor (10-min peak) was not affected by actinomycin D.

The newly synthesized protein was identified as a specific receptor by its high affinity for [^{3}H]-cortisol and by the ability of this complex to stimulate the activity of RNA polymerases of isolated rat liver nuclei, as judged by [^{3}H]-UTP incorporation into nuclear RNA.

The data therefore indicate that cortisol controls the rate of synthesis of its specific receptor protein.

Our results are in good agreement with data from other laboratories, relevant to the mode of action of sex hormones. Thus several groups have reported the induction of specific cytoplasmic protein in rat uterus at very early times after administration of estradiol. Many explanations were given for the function of this protein[33–37].

There are few data indicating that the number of sex steroid receptor molecules may also be hormonally regulated. Thus, estradiol provokes the synthesis of its receptor in the prepubertal rat uterus[37] and estrogens stimulate the progesterone receptor synthesis[38]. This increase appeared to be due to new receptor synthesis, since actinomycin D and cycloheximide blocked this response[39]. It is suggested that the transfer of the estradiol receptor complex from cytosol to the nuclear compartment is responsible for an increase of the synthesis of cytosol receptor[40].

Our findings that cortisol dependent "*de novo*" synthesis of its receptor protein, even within the first 10 min after cortisol administration to the animals, seems to be of special interest for a better understanding of the mode of cortisol action on gene expression. It might indicate that cortisol activates the rate of translation of preformed mRNA "coding" receptor protein very early after administration, or stimulates the mobilization of this mRNA from soluble mRNP-pool into the polysomes. The existence of a pool, i.e. of stored inactive mRNA in the cytoplasm and a possibility that it can be mobilized into active polyribosomes, following a hormonal or nutritive signal, has often been considered, but this has been difficult to prove[41–43].

A rise in receptor synthesis, observed 10 min after cortisol administration, cannot be attributed to the translation of *de novo* synthesized specific mRNA coding receptor protein[43], but rather to some other mechanism(s), such as the acceleration of translation of preformed specific mRNA, or to faster incorporation of specific mRNA from cytoplasmic "pool" of mRNA particles into polysomes, or to modifications of ribosomes and/or translation protein factors. Our results indicate that polysomes and the S-100 fraction from cortisol treated animals show increased endogenous mRNA-directed protein synthetic activity as compared to controls. Furthermore, we found that cortisol treatment increases the ability of S-100 fraction and polysomes to translate poly-U. Cortisol induced enhancement of poly-U translation suggests that the changes in the activity of S-100 fraction and polysomes are associated with modifications of ribosomes and/or protein, factors, probably transferases I and II, involved in the process of elongation. This therefore may suggest that cortisol can exert the initial effect at the level of selective and specific translation of some pre-existing mRNA's.

Our results relevant to the modification of protein synthesizing capacity are in good agreement with data from other laboratories. Thus, when protein synthesis has been studied in cell free systems, the capacity of *Xenopus* liver ribosomes to incorporate amino acids into protein was found to be enhanced by estrogen administration *in vivo*[44, 45].

The data described support the concept of our working hypothesis. They indicate that the overall protein synthetic capacity of target tissues is markedly enhanced immediately after administration of steroids. Glucose induction of insulin synthesis may be of interest as a support of our concept. These data indicate that the increased biosynthesis of insulin, in response to high glucose, occurs rapidly and does not initially require synthesis of new RNA or protein. Thus, a major effect of glucose appears to be a specific and selective stimulation of the rate of translation of pre-existing proinsulin mRNA[46].

It becomes evident from our studies that some factor(s) essential for the specific translation of receptor protein messenger must be activated during the process of formation of hormone specific receptor complex. What these factor(s) are or whether they act directly on the translation machinery is not certain, but it may be a combination of more than one of the following: subunit(s) of the receptor system, released after the receptor–hormone complex formation, may directly or indirectly activate ribosomes and/or factors of translation initiation and/or elongation; cortisol may activate the factor(s) for the selective control of translation of cortisol receptor protein mRNA in the liver like for example "capping" factors[47]. From our studies is also evident that the total integrated response to the hormone-full course of steroid hormone action is not only due to the modification of the transcription rate of specific genes, but that it also involves, in a eucariotic cell, very early enhancement of translation of some preformed mRNA, as well as the modification of pre-existing proteins.

The major cortisol-mediated early effects observed in our experiments are: the enhancement of the synthesis of cortisol specific receptor; modifications of

some factors of translatory machinery; and the enhanced turnover and phosphorylation of histones and nonhistone proteins in target cells. Our data provide evidence that all these immediate events in the rat liver cells are dependent and regulated by cortisol, but it remains to be elucidated whether cortisol induces these events as a primary response, directly or indirectly. We still have no answer to these questions, but our data suggest that the steroid receptor may act as a receptor system "encoded" with a defined metabolic program.

What is the biological relevance of these early events dependent on the receptor system activation?

There is no direct answer to this question. It is possible that these very early events, such as phosphorylation and acetylation of regulatory proteins as well as the early translation of specific and selective mRNA, are involved in generating substantial changes in chromatin that appear to underlie the specific gene activation, i.e. the selective transcription of a few specific structural genes.

It is difficult to reconcile this large number of early events, presumably induced by receptor activation, with the expression and activation of a limited number of specific genes in target cells. This discrepancy might be resolved by assuming that chromatin structure changes, largely non-specific, are necessary to expose the "acceptor" sites. The energy requiring conformational changes might underlie in the recognition and binding to the "acceptor" sites of receptor "steroidophilic" component carrying hormone. It is intriguing in this regard that the progesterone receptor appears to bind ATP[48], implying, perhaps, its involvement in chromatin phosphorylation. Moreover, steroid induced protein phosphatase activity has recently been reported in the systems responsive to estrogen[49] progesterone[50], and aldosterone[51].

CONCLUSIONS

While the exact mechanism(s) of steroid hormone action in the respective target cells and tissues is still not elucidated, our current knowledge about steroid receptors and biological responses in the responsive target cells suggests a general concept of steroid action, which might be extended to explain the mechanism of action of all steroids.

In an attempt to integrate both the immediate extragenomic and genetic effects of steroid action, we propose an integrated concept of steroid action as a working hypothesis, which remains to be experimentally explored.

The key stone of this hypothesis is the structure and function of steroid receptors. Considering the extragenomic and genomic responses to steroids in different target cells and tissues it appears logical to propose that a native steroid receptor is a specific macromolecular system (multimer), which consists of several specific regulatory—subunits, comprising a defined program (code) for multiple cooperative metabolic functions that underlie the expression of specific genes. The binding of steroids to specific receptor, occurring in the cytoplasm of target cells, initiates the activation of the receptor-system which results in a dissociation and dissagregation of the receptor-system into the subunits. The free subunits of the receptor-system exert their effects at different levels of the cells' regulatory machinery. The "steroidophilic" subunit (subunits binding steroids) is translocated in the cell nuclei and it interacts with the acceptor sites of the target chromatin. Other subunits, which may not bind steroids, can, by still unknown mechanism(s), modify certain classes of existing proteins, involved probably in the regulation of translation and of conformational state of the membrane system(s). The conformational transitions of membrane proteins might activate protein kinases. It might stimulate phosphorylation of the existing protein constituents of genomic and extragenomic regulatory mechanisms. The modifing action of receptor subunits upon the existing regulatory mechanisms underlies extragenomic immediate effects of steroids, which occur at time intervals too short to be accounted for by the selective expression of the activated genes. The late effects of steroids, namely the selective expression of a series of structural genes in target cells, could be explained by the regulatory function of "steroidophilic" subunit of the receptor-system at the level of transcription. The subunit of receptor which binds the respective steroids, i.e. a "steroidophilic" subunit appears to act as a key stone in the formation of the macromolecular receptor system, whose molecular stability and metabolic functions are regulated by the presence of steroid hormones.

In the proposed hypothesis the activation of the native receptor-system is a crucial event in the full course of steroid hormone action. It may trigger not only the selective expression of specific genes in eucariotic cells, but also a series of effects seen in the target cells and tissues at time intervals much shorter than those accounted for by the selective gene expression.

What the biological relevance of these acute changes to the specific gene expression is we do not know.

Whether or not they enter into the full course of the steroid hormones specific action upon gene expression remains to be explored experimentally; nevertheless, it seems quite probable from the available data that they do. In the course of our studies several important questions were posed: whether or not the mRNA coding receptor is subjected to selective translational control; whether or not protein kinases are activated by disaggregation of the receptor system. These and other major questions still have to be resolved. It is evident that we can gain a better insight into the mechanism of steroid action only by improving our understanding of the interactions of macromolecules in nucleochromatin, translatory and membranes systems.

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