Effect of Cortisol \textit{in vivo} on Amino Acid Incorporation into Protein by Rat Liver Ribosomes \textit{in vitro}

SABURO OMATA AND SHOGO ICHII

Division of Physiology, Institute of Steroid Research, Tottori University School of Medicine, Yonago-shi

Synopsis

Effect of cortisol administration \textit{in vivo} on the activity of protein synthesis in ribosomal and polysomal preparations from rat liver was examined. Purified ribosomes obtained from adrenalectomized and cortisol-treated rats showed a higher activity of amino acid incorporation in the presence of poly U \textit{in vitro} than those from adrenalectomized, untreated rats. Higher activity of poly U-directed amino acid incorporation in ribosomes from the hormone-treated animals was retained even after elimination of endogenous m-RNA by preincubation, and therefore, it was concluded that the activity of ribosomes was altered by cortisol-treatment.

Significant increase in heavy polysomes was observed 4 hr after administration of the hormone. Purified polysomes were fractionated on sucrose gradient, and the analysis of the fractions for amino acid incorporating activity revealed that light polysomes obtained from cortisol-treated rats were twice as active as those from control animals, while no significant difference was detected in the activity of heavy polysomal fractions.

Extensive studies have been made on the glucocorticoid-induced changes in the synthesis of RNA (Kenney, \textit{et al.}, 1965; Tata, 1966; Kidson, 1967; Drews and Brawerman, 1967), and considerable evidence has accumulated suggesting that glucocorticoids regulate gene expression at the site of transcription (Dahmus and Bonner, 1965; Greenman \textit{et al.}, 1965; Stackhous \textit{et al.}, 1968; Jacob \textit{et al.}, 1969). On the other hand, little is known for the effect of the hormone on the translational process of gene expression. In the liver of rat, increase in RNA synthesis (Feigelson \textit{et al.}, 1962) and activation of nuclear RNA polymerase (Barnabei \textit{et al.}, 1966) after glucocorticoid administration was followed by the synthesis of specific proteins (Kenney \textit{et al.}, 1965; Kenney \textit{et al.}, 1967). Although it has been shown that increase in polysomes in the liver was mediated through glucocorticoids (Koike \textit{et al.}, 1967; Cammarano \textit{et al.}, 1968), many conflicting results have appeared concerning to the effect of the hormone on the capacity of labelled amino acids incorporation into proteins by the cell-free system \textit{in vitro} (Leon \textit{et al.}, 1962; Leon, 1966; Pena \textit{et al.}, 1966; Venuto and Lange, 1967a, 1967b; Koike \textit{et al.}, 1967). Studies on a primary and specific effect of glucocorticoids on liver cytoplasmic system seem to be of considerable interest, in a view of the recent hypothesis of hormone action at the site of translational process (Tata, 1968). We have investigated the changes in amino acid incorporation \textit{in vitro} by ribosomes from the livers of rats after administration of cortisol.

Materials and Methods

\textit{Reagents}: \(14^C\)-Phenylalanine (L-phenylalanine-\(14^C\), uniformly labelled, specific activity 459 mC/mM) was purchased from the Radiochemical Centre, Amersham, England. \(14^C\)-labelled chlorella hydrolyzate (specific activity, 5.28 mC/mM carbon) was a gift from the Institute of Applied Microbiology.

Received for publication October 1, 1969.
University of Tokyo, Japan. ATP, GTP, CTP, poly U (polynucleotidic acid), and cortisol were obtained from the Sigma Chemical Company, St. Louis, Mo., and phosphoenol pyruvic acid (sodium salt) and pyruvate kinase (E.C 2.7.1.40) from Boehringer and Soehne GmbH., Mannheim, Germany.

**Animals:** Male Wistar rats weighing 230 - 260 g were used. They were fed ad libitum with balanced diet (Funabashi Farms, Ltd., Chiba, Japan) and kept in a room of constant temperature (27°C) and humidity (60%) with a 8 a.m. - 8 p.m. daylight cycle. All animals were bilaterally adrenalectomized and given 1% NaCl thereafter. They were fasted for 18 hr prior to sacrifice. Cortisol was suspended in 0.9% NaCl with the aid of Tween 80 and injected intraperitoneally (5 mg/100 g body weight).

**Preparation of ribosomes:** The rats were killed by exsanguination, their livers removed immediately and chilled in ice-cold 0.25 M sucrose in medium A (10 mM MgSO4, 80 mM KCl, 5 mM β-mercaptoethanol, 50 mM Tris-HCl, pH 7.8). Livers from rats were pooled and homogenized with 2.5 volumes of ice-cold 0.25 M sucrose in medium A with teflon-glass homogenizer. Homogenate was centrifuged for 10 min at 15,000 g and the post-mitochondrial fraction was spun further for 90 min in an International A-170 rotor at 78,000 g. Ribosomes were purified from the resulting pellet with the method of Takanami (1960) as modified by Tashiro and Siekevitz (1965), except that sodium deoxycholate (1%) and Lubrol WX (0.5%) were used as detergents.

**Preparation of polysomes:** Polysomes were obtained from the post-mitochondrial supernatant of the liver homogenate essentially according to Wittman et al. (1969) with slight modifications described below. Post-mitochondrial supernatant fraction was mixed successively with Lubrol WX and sodium deoxycholate to give a final concentration of 0.5% and 1%, respectively. Detergent-treated post-mitochondrial fraction was spun over the discontinuous sucrose gradient and centrifuged in an International SB 283 rotor at 150,000 g for 4 hr. The transparent pellet was suspended in a small volume of medium A.

**Incorporation of 14C-labelled amino acids in vitro:** The complete amino acid incorporation system consisted of the following components in the final volume of 1.0 ml: 40 mM Tris-HCl buffer, pH 7.8, 80 mM KCl, 15 mM MgSO4, 1 mM ATP, 0.4 mM GTP, 0.2 mM CTP, 10 mM phosphoenol pyruvate, 60 μg pyruvate kinase, 10 mM β-mercaptoethanol, 100 μg polyuridylic acid, 0.5 μCi 14C-labelled phenylalanine or chlorella hydrolyzate, 100 - 200 μg protein equivalent of ribosomes, pH 5 fraction (Hoagland et al., 1958) or dialyzed 105,000 g supernatant (Parthier et al., 1967) from adrenalectomized rats, containing 2.5 - 3.5 mg proteins. ATP, GTP, CTP, and phosphoenol pyruvate were neutralized before addition to the incubation tubes. Incubation was carried out at 37°C with a gentle shaking for indicated time intervals. Incubation was terminated by adding 3 ml of 10% trichloroacetic acid (TCA) containing 0.2% nonlabelled phenylalanine or amino acid mixture. The precipitate was washed twice in the cold 5% TCA and extracted twice at 90°C for 15 min with the same solution then washed once with ethanol, followed with ether. The precipitate was dried and dissolved into concentrated formic acid. An aliquot of this solution was taken into scintillation vial, mixed with a small amount of concentrated perchloric acid and hydrogen peroxide, and oxidized at 70°C for 20 min. After cooling, 5 ml of cellosolve and 10 ml of the scintillator fluid were added (Mahin and Lofberg, 1966), and the radio-activity was determined in a liquid scintillation spectrometer. The quenching was corrected for individual samples with the use of internal standard. The activity of the incorporation was expressed as counts per minute per 100 μg ribosomal protein. Protein was estimated by the method of Lowry et al. (1951).

**Sucrose density gradient centrifugation of ribosomes and polysomes:** Post-mitochondrial supernatant fraction prepared as described above was mixed with sodium deoxycholate to give a final concentration of 1.2%. The detergent-treated fraction was layered over 32 ml of 10 - 30% (w/w) linear sucrose gradient in medium A and centrifuged in an International SB 110 rotor at 70,000 g for 3 hr at 4°C. Purified ribosomes were layered over 12.5 ml of a linear 10 - 35% (w/w) sucrose gradient in medium A and centrifuged in an International SB 283 rotor at 100,000 g for 3 hr at 4°C. Conditions for the gradient centrifugation of purified polysomes were essentially the same as for the ribosomes, except that the gradient was spun at 70,000 g for 2.5 hr. After centrifugation, the gradient was analyzed with the ISCO density gradient fractionator with a automatic recorder at a constant scanning speed of 2 ml/min using 1 cm light path-flow-cell.

**Results**

**Sedimentation profiles of post-mitochondrial supernatant fraction of livers from rats received cortisol injection**

Figure 1 indicates distribution patterns of ribosomes in post-mitochondrial supernatant fractions obtained from livers of rats at various intervals after cortisol administration. Imme-
Fig. 1. Changes in distribution of hepatic ribosomes after treatment with cortisol. Rats were sacrificed at various times after intraperitoneal injection of cortisol. Control rats were injected vehicle alone and sacrificed 4 hr later. Livers from 3 rats were pooled and treated as described in Materials and Methods.

Fig. 2. Changes in distribution of polysomes in the post-mitochondrial supernatant fractions at various times after cortisol treatment. The quantitative analysis was made on the area under the profiles obtained in Fig. 1. Ratio of polysomes (aggregates larger than trimers) to that of monomers plus dimers was plotted as a function of the time after cortisol injection. The ratio at zero time refers to the value for the control animals.

Immediately after the hormone treatment, polysome region was reduced and recovered to the control level at one hour later. A significant increase in heavier polysomes with a concomitant decrease of the monomer peak was observed at 4 hr after the hormone treatment. This results is in agreement with that of Cammarano et al. (1968). Eight hours after the treatment distribution pattern of ribosomes was almost identical to that of control animals. Polysome area (aggregates larger than trimers) was measured quantitatively and its changes after the hormone treatment were depicted in Figure 2.

On the basis of the results shown in Figures 1 and 2, in the following experiments ribosomes and polysomes were prepared from the livers 4 hr after cortisol treatment, since there were close correlations between the ribosome assembly and protein synthetic activity in hepatic cells (Gierer, 1963; Sox and Hoagland, 1966; Blobel and Potter, 1967).
14C-Phenylalanine incorporation in vitro by ribosomes obtained from livers of control and cortisol-treated rats

Time-course of the incorporation of 14C-phenylalanine by ribosomes from control and the hormone treated rats in the presence or absence of poly U is depicted in Figure 3. The incorporation was almost linear for 20 min, and proportional to the concentration of ribosomes in the incubation mixture. A very little incorporation was found in the absence of poly U by the ribosomal preparations. Ribosomes from the livers of adrenalectomized and cortisol-treated rats showed an higher activity of the incorporation than those from adrenalectomized ones. However, there seems to be a possibility that the observed difference in amino acid incorporating activity between ribosomes from the control and the cortisol-treated rats is attributable to the difference of m-RNA attached to the ribosomal preparations. Therefore, in the subsequent experiments, purified ribosomes were preincubated for 50 min at 37°C in a complete amino acid incorporating system except 14C-labelled amino acid and poly U to minimize attached m-RNA according to the description of Parthier et al. (1967), and the ribosomes were further incubated in the complete amino acid incorporating system. It is clear from the results presented in Figure 4 that the preincubation did not abolish the effect of cortisol-treatment on the incorporating activity. A single peak was obtained from the sucrose gradient of the preincubated ribosomes (Fig. 5). Increase in amino acid incorporating activity by the hormone-treatment of the rats was not so remarkable, but results were consistent and reproducible in the separate experiments. It is concluded that amino acid incorporating activity of ribosomes in vitro is enhanced by cortisol treatment in vivo, and that this may be ascribed to the altered properties of the ribosomes per se.
Amino acid incorporation in vitro by polysomes from the livers of control and cortisol-treated rats

Sedimentation profiles of purified polysomes derived from the livers of cortisol-treated rats was essentially similar to that from control rats, although amount of polysomes per weight of the tissue was greater in the cortisol-treated rats.

The rate of amino acid incorporation into protein in the sucrose gradient fractions of purified polysomes was determined and most activity was found in larger aggregates (Fig. 6). However, when the incorporated radioactivity is computed per unit of RNA of ribosomes (per optical density at 260 mg), it is apparent that the lighter polysomal fractions are more active than the heavier ones. The effect of cortisol was obviously observed in the lighter fractions but not in the heavier ones (Fig. 6).

Discussion

Leon et al. (1962) reported that the increase of the protein synthetic activity of cell-free liver system at 10-16 hr after administration of natural or synthetic glucocorticoids to rats was ascribed to the higher activity of microsomal fraction. Similar results were also obtained by Pena et al. (1966), Leon (1966) and Koike et al. (1967), using the intact animals. On the other hand, when ribonucleoprotein particles from livers of adrenalectomized rats were used, the rate of amino acid incorporation into proteins was more than twice of that of the particles from the intact rats (Venuto and Lange, 1967a) and the elevated activity of the particles was returned to the normal level by injection of corticosterone to the adrenalectomized rats. The same authors (Venuto and Lange, 1967b) also reported that the amino acid incorporation by ribonucleoprotein particles from the livers of adrenalectomized rats was higher for the initial 15 days, and then decreased below the control level between 21st and 31st days after operation.

Fig. 4. Poly U-directed $^{14}$C-phenylalanine incorporation by purified and preincubated ribosomes. Ribosomes were preincubated as described in the text, and then assayed for the activity of $^{14}$C-phenylalanine incorporation. Closed circles with solid line represent ribosomes from the livers of control rats and open circles with dotted line, from cortisol-treated rats.

The results obtained by Koike et al. provided an another complex feature; amino acid incorporation in cell-free systems from the livers of mice was affected by cortisone administration in vivo, but not by cortisol (Koike et al., 1967).

Conflicting results from these laboratories might not be wholly explicable with the different experimental conditions used. These authors used the total ribosomes, microsomes, or post-mitochondrial fractions for the assay of in vitro protein synthetic activity. Therefore, results obtained from these experiments may not provide direct information on the translational control of protein synthesis by glucocorticoids. The amino acid incorporation of these preparation in vitro depends completely on the presence of endogenous m-RNA (Parthier et al., 1967; Weksler and Gelboin, 1967) or addition of synthetic m-RNA (Korner, 1966; Smuckler et al., 1968; Staehlin, 1969).
Fig. 5. Sedimentation profiles of purified and preincubated ribosomes.
(a) Ribosomes from control rats.  (b) Ribosomes from cortisol-treated rats. (4 hr)

Fig. 6. Amino acid incorporation into protein by the purified polysomes.
Purified polysomes were centrifuged on a linear sucrose gradient as in the text. Fractions of 1.0 ml were collected by ISCO density gradient fractionator, and each fraction was assayed for amino acid incorporation and optical density at 260 m\text{\textmu}.

Livers from 3 rats were pooled for the preparation of polysomes. Closed circles with solid line represent optical density of the fraction at 260 m\text{\textmu} and open circles with dotted line, amount of radioactivity in the fraction. Specific incorporating activity of the fraction (incorporated radioactivity per optical density at 260 m\text{\textmu}) is expressed as triangles with broken line.

(a) control rats  (b) cortisol-treated rats
The present results demonstrated that glucocorticoid administration in vivo significantly enhanced the amino acid incorporating activity of ribosomes. The elevated incorporation due to cortisol administration was also observed in the purified, preincubated ribosomes using poly U as the template. Therefore it seems likely that cortisol induced some alterations in the capacity of amino acid incorporation of ribosomes. Garren et al. (1967) also found the decreased activity in poly U-directed phenylalanine incorporation of single ribosomes isolated from the liver of hypophysectomized and thyroidectomized rats and this was interpreted as that pituitary and thyroid hormones influence the protein synthesis on the liver at the translational level. The precursor incorporation into liver RNA fractions was accelerated within 4 to 6 hr after glucocorticoid administration (Feigelson, et al., 1962), and increased polysome fraction and elevated capacity of the amino acid incorporation in single ribosome were observed at the same period after cortisol administration in this experiment. Therefore, an early action of glucocorticoids may reside both in nucleus and cytoplasm of hepatic cells.

Significance of ribosomal sub-unit exchange during protein synthesis has been reported in the mammalian system (Colombo et al., 1968; Hogan, 1969) as well as in the bacterial system (Kaempfer, 1968), and Martin and Wool found that effect of insulin in vivo on the protein synthetic activity of 80 s ribosomes from muscle in vitro was restricted to the 60 s sub-unit. The nature of glucocorticoid-induced alteration in ribosomes observed in this experiment is to wait for further analysis.

Assays for amino acid incorporating activity of purified polysome fractions revealed that ribosomes on the light polysome fractions obtained from the livers of cortisol-treated rats were nearly twice as active in protein synthesis as those from control animals. However, “specific activity” (see Fig. 6c) of ribosomes on the heavier polysomes was lower than that on the lighter ones in this experiment, and this is not in agreement with those reported by Noll et al. (1963) and Sox and Hoagland (1966) in which ribosomes on the heavier polysomes possessed higher activity of protein synthesis. This discrepancy is difficult to explain at present moment.

References

Tashiro, Y. and P. Siekevitz (1965) J. Mol. Biol. 11, 149.