Effects of Corticosterone on Phosphorylation of Rat Liver Nuclear Proteins *in Vitro*[1] (37532)

G. D. BOTTOMS AND R. A. JUNGMANN[2]
(Introduced by E. V. Morse)

Department of Physiology and Pharmacology, School of Veterinary Science and Medicine, Purdue University, Lafayette, Indiana 47907

Intracellular action sites of glucocorticoids have been studied for many years, but their precise action is still being pursued. One of the very early events that occurs after corticosteroids are injected into animals is the association of the steroid with specific cytoplasmic receptors, followed by a temperature dependent modification of the cytosol-receptor complex which permits nuclear binding of the steroid and altered RNA synthesis (1–4, 9, 12, 14).

Exactly how the steroid-receptor complexes alter nuclear RNA synthesis is unknown. It is widely accepted that nuclear proteins assist in the regulation of genetic expression (5, 10). The role of nuclear acidic proteins in the sequence of events after hormone treatment is of particular interest since it has been observed that synthesis of certain nuclear acidic proteins are selectively stimulated by cortisol (13) and estradiol (15). Also, nuclear acidic proteins are known to be highly concentrated in metabolically active tissue (6) and localized in regions of active RNA synthesis (8).

Phosphorylation of nuclear acidic proteins seems to be a critical variable in the interactions between these proteins and DNA, leading to altered RNA synthesis. The phosphorylation of liver nuclear acidic proteins is altered when cortisol stimulates RNA synthesis in the liver and may be an early obligatory action of cortisol (16).

The work reported here tested the possibilities whether or not one of the very early effects of corticosterone treatment in rats is altered phosphorylation of nuclear proteins and whether cytosol steroid-receptor complexes are necessary for altered phosphorylation of nuclear proteins.

Materials and Methods. Animals. Male Sprague–Dawley rats, 200–300 g, which had been adrenalectomized 3–5 days before, were used in all of the experiments. In order to determine the *in vivo* effects of corticosterone, rats were injected (ip) with 1 mg corticosteroid/kg body weight and control rats were injected with the same volume of saline. They were killed 1.5 hr after treatment and the livers were perfused with heparinized saline.

Isolation of nuclei and cytosol. Nuclei were isolated by a procedure similar to that reported by Widnell and Tata (17). Minced tissues were homogenized in 2 volumes (w/v) of 0.32 *M* sucrose, 3 m*M* $CaCl_2$, 0.05 *M* Tris buffer, pH 7.4, in a Potter–Elvehjem homogenizer. The homogenate was filtered through gauze and again homogenized with five strokes in an all glass homogenizer. The homogenate was then centrifuged at 800*g* for 10 min. The 800*g* supernatant was centrifuged at 100,000*g* for 1 hr in order to obtain the cytosol (100,000*g* supernatant) fraction. The crude nuclear pellet was resuspended in 2.4 *M* sucrose and centrifuged at 100,000*g* for 1 hr. The purified nuclei were resuspended in 0.25 *M* sucrose, 3 m*M* $MgCl_2$, 0.05 *M* Tris buffer, pH 7.4. Microscopic examination showed that the nuclei were intact and essen-

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[2] Department of Biochemistry, Northwestern University Medical School, Chicago, Illinois 60611.

tially free from cytoplasmic contaminants and cells.

Interaction of nuclei with corticosterone-receptor complex and protein kinase assay. Nuclei from 10 g of liver were divided into 4 equal aliquots and incubated at 37° for 10 min with one of the following: (a) cytosol, (b) cytosol previously incubated for 1 hr at 4° with 10^{-7} *M* corticosterone, (c) buffer, or (d) buffer + 10^{-7} *M* corticosterone. After incubation nuclei were washed with 0.25 *M* sucrose–0.05 *M* Tris buffer, resuspended in 2.4 *M* sucrose, and sedimented at 100,000*g* for 1 hr.

The nuclear pellets were resuspended in 0.25 *M* sucrose, 3 m*M* $MgCl_2$, 0.05 *M* Tris buffer, pH 7.4 so that each ml of buffer contained nuclei from about 2 g of liver. A 0.2 ml aliquot was saved for protein determination and four 0.2 ml aliquots were transferred to small test tubes. After addition of two μCi of [γ-^{32}P]ATP to each tube, they were incubated at 37° for 5–30 min. After incubation, the reactions were stopped by the addition of ice-cold 16% tricholoroacetic acid (TCA). The reaction in one tube of each group was stopped with TCA at time zero. The counts per minute in these tubes were used as background. The samples were filtered over Millipore filters and washed with TCA. The filters were placed in liquid scintillation vials containing 10 ml of Insta Gel,[3] and the ^{32}P activity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

Results are expressed as the average of triplicate observations made on 5 groups of rats. A two-way analysis of variance was made and the treatment means were compared by Newman–Keuls sequential range test.

Extraction of nuclear proteins. Histones and nuclear acidic proteins were isolated and separated on polyacylamide gels by the method reported by Teng *et al.* (16). Densitometer tracings were obtained by scanning the gels in a Gilford model 2000 spectrophotometer. Duplicate gels were sliced. The radioactivity was determined in the slices from one gel after digestion with protosol. Protein was measured in the corresponding gel slices of a duplicate gel at 600 nm after elution of the dye with dimethylsulfoxide.

[3] Packard Instrument Co., Inc., Downers Grove, Ill. 60515.

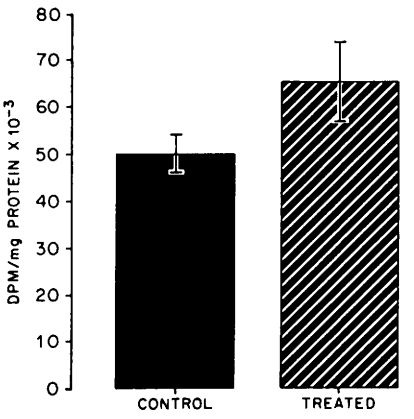

FIG. 1. Effects of corticosterone treatment on ^{32}P incorporation into TCA-precipitable material in isolated liver nuclei incubated for 15 minutes with [γ-^{32}P]ATP. Results are the average of duplicate determinations made on 4 rats ± SE.

Results. The effects of corticosterone injection (1 mg/kg body weight, ip) on the uptake of ^{32}P by isolated liver nuclei are shown in Fig. 1. A significant ($p < 0.05$) increase in the incorporation of ^{32}P in the TCA precipitable material of nuclei isolated from treated rats was observed.

In order to determine if cytoplasmic steroid-receptor molecules are involved in the physiological actions of glucocorticoids, the following experiments were conducted. Liver nuclei were isolated from adrenalectomized rats and incubated for 10 min with (a) buffer, (b) buffer containing 10^{-7} *M* corticosterone, (c) liver cytosol, (d) liver cytosol previously incubated with 10^{-7} *M* corticosterone. These nuclei were cleaned of most cytoplasmic contaminants and incubated with [γ-^{32}P]ATP for 5–30 min. After incubation, the amount of ^{32}P present in the TCA insoluble fraction was determined and is shown in Fig. 2. Free steroid in the buffer had no significant effect on phosphorylation in nuclei when compared to nuclei incubated with buffer alone. Phosphorylation in nuclei previously incubated with cytosol (without added corticosterone) was significantly greater ($p < 0.05$) than that observed in nuclei previously incubated

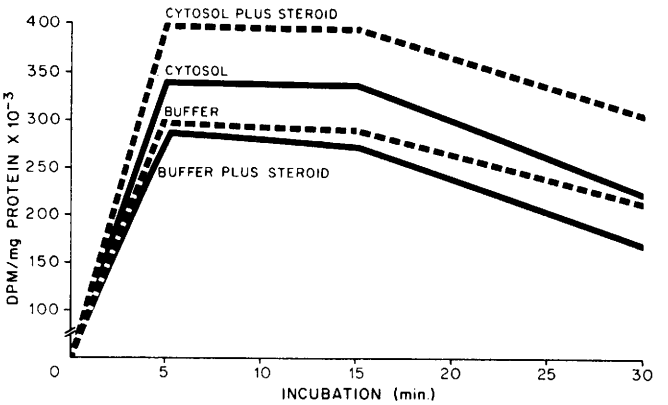

FIG. 2. ^{32}P-incorporation into TCA-precipitable material in liver nuclei previously incubated with (a) cytosol plus 10^{-7} *M* corticosterone, (b) cytosol, (c) buffer, or (d) buffer plus 10^{-7} *M* corticosterone. Results are the average of duplicate determinations made on 4 rats ± SE.

with buffer or buffer plus free corticosterone. Nuclei, incubated with cytosol which had been previously incubated with 10^{-7} *M* corticosterone, incorporated significantly more ^{32}P than nuclei incubated with cytosol ($p < 0.05$), or buffer plus 10^{-7} *M* corticosterone ($p < 0.01$) or buffer alone ($p < 0.01$). Maximum incorporation had been reached during the first 5 min with no further change up to 15 min. Incubation for longer than 15 min resulted in a loss of ^{32}P counts in all groups (Fig. 2).

In order to determine if the increased incorporation of radioactivity was in the protein fraction rather than nucleic acids, TCA-precipitated samples were heated at 90° for 20 min to hydrolize nucleic acids in this fraction. The precipitate was then collected on Millipore filters and the radioactivity determined. A similar difference was observed between control and treated rats.

Additional experiments were performed in order to determine if the increased phosphorylation, observed in liver nuclei previously incubated with cytosol containing 10^{-7} *M* corticosterone, was specific for liver nuclei. Thymus nuclei isolated from adrenalectomized rats were incubated with (a) thymus cytosol, (b) thymus cytosol plus corticosterone, (c) liver cytosol, and (d) liver cytosol plus corticosterone. The results are shown in Fig. 3. No significant difference in phosphorylation was observed in thymus nuclei previously incubated with liver cytosol contain-

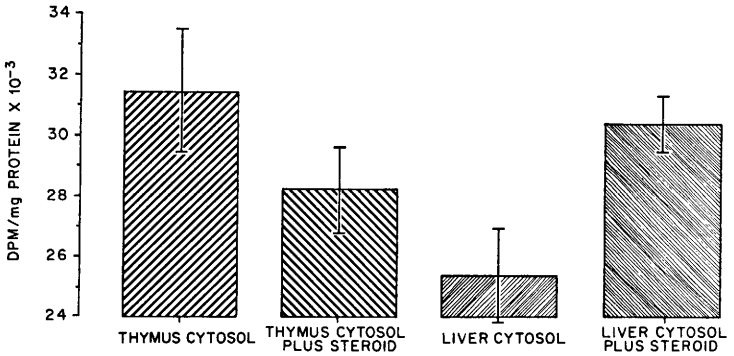

FIG. 3. ^{32}P-incorporation into TCA-precipitable material in thymus nuclei previously incubated with (a) thymus cytosol, (b) thymus cytosol plus 10^{-7} *M* corticosterone, (c) liver cytosol, or (d) liver cytosol plus 10^{-7} *M* corticosterone. Results are the average of duplicate determinations made on 4 rats ± SE.

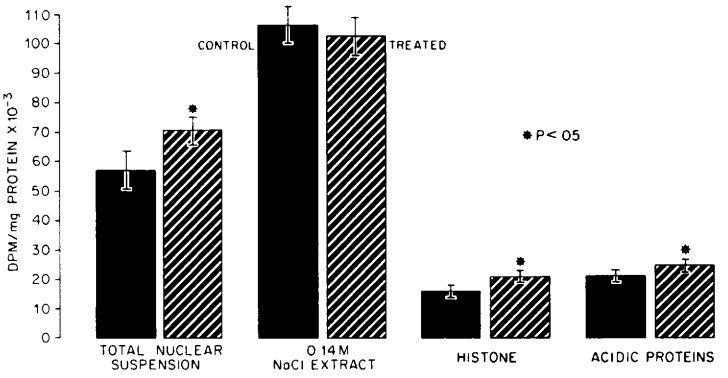

FIG. 4. Effects of corticosterone treatment on phosphorylation of nuclear proteins in isolated nuclei incubated with [γ-^{32}P]ATP. Results are the average of duplicate determinations made on 4 rats ± SE.

ing 10^{-7} *M* corticosterone compared to those incubated with thymus cytosol or thymus cytosol containing 10^{-7} *M* corticosterone. Phosphorylation was significantly greater ($p < 0.01$) in thymus nuclei incubated with liver cytosol plus 10^{-7} *M* corticosterone compared to thymus nuclei incubated with liver cytosol alone.

The results of attempts to localize the increased phosphorylation in nuclear subfractions are shown in Fig. 4. Corticosterone treatment of rats significantly increased phosphorylation of nuclear histones and acidic proteins. There was no significant difference of ^{32}P incorporation into protein of the 0.14 *M* NaCl extracts or of the lipid extract.

Effects of corticosterone treatment on the phosphorylation of specific nuclear acidic protein bands are shown in Fig. 5. Treatment resulted in a significantly increased phosphorylation of proteins in section 4 of the gel.

The specific activity of each histone band was also determined. No significant effect of corticosterone treatment on the phosphorylation of any histone band was observed. Only slightly increased phosphorylation was observed in each band.

Discussion. These experiments were conducted in order to test the hypothesis that one of the early affects of glucocorticoids in the liver is altered phosphorylation of nuclear protein. It was found that either treatment of rats with corticosterone (Fig. 1), or incubation of isolated nuclei with cytosol containing 10^{-7} *M* corticosterone (Fig. 2), significantly increased incorporation into isolated nuclei. A significant increase was observed in TCA-precipitable material, histones, and acidic proteins (Figs. 1 and 4) of rats previously injected with corticosterone. Similar results were observed when nuclei were previously incubated with cytosol containing 10^{-7} *M* corticosterone.

The data indicate that increased phosphorylation of nuclear proteins is one of the very early events that occur after corticosterone treatment. A physiological role of cytoplasmic steroid-receptor complexes is indicated by the observation that incubation of nuclei with cytosol containing 10^{-7} *M* corticosterone significantly increased phosphorylation in nuclear subfractions when compared to nuclei incubated with cytosol alone (Fig. 2). An obligatory role of the cytosol steroid-receptor complex is suggested since nuclei incubated with 10^{-7} *M* corticosterone alone were not affected (Fig. 2).

These data support previously reported evidence for a role of cytoplasmic steroid-protein complexes on RNA polymerase activity in the liver (6) and hypothalamus (14). Similar results were reported by Beato *et al.* (3).

Doenecke *et al.* (7) reported no effect on phosphorylation of nuclear proteins after incubation of nuclei with cortisol alone. We also found no effect on phosphorylation in isolated nuclei incubated with corticosterone alone, but phosphorylation was increased in nuclei previously incubated with cytosol con-

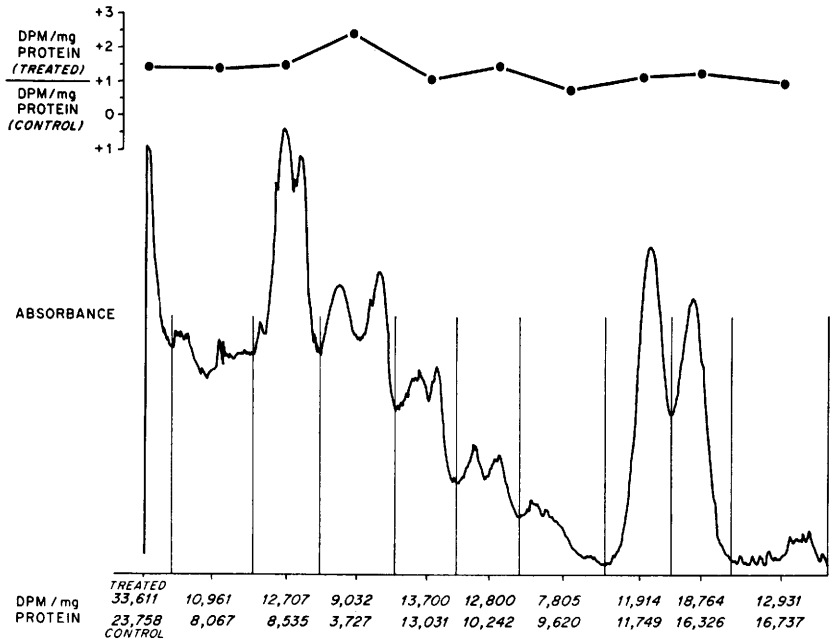

FIG. 5. Densitometer tracing and analysis of the ^{32}P-labeling pattern of liver nuclear acidic proteins separated on polyacrylamide gels. Each value represents the average of 4 experiments. Adrenalectomized rats were treated with 1 mg of corticosterone/kg body weight, or saline 1.5 hr before killed. Nuclei from 20 to 25 g of liver were incubated with [γ-^{32}P]ATP for 15 min. Acidic proteins were isolated and separated by polyacylamide electrophoresis as indicated under Materials and Methods.

taining 10^{-7} *M* corticosterone (Fig. 2).

The liver cytosol steroid-protein complex is capable of stimulating increased phosphorylation in thymus nuclei as well as liver nuclei (Figs. 2 and 3). It is possible that specific genes in the liver are activated by corticosterone due to the presence of the cytosol receptor. These same genes in the thymus may not be activated by the hormone since the thymus does not contain a specific cytoplasmic receptor similar to that found in the liver.

Teng *et al.* (15) reported that the kinetics of ^{32}P incorporation into individual proteins of the phenol extract of rat liver nuclei were also altered by previous treatment with hydrocortisone. The concentrations they reported are at least 20–40 times greater than physiological levels and must be considered pharmacological levels. Our data are consistent with their results, but physiological levels of corticosterone were used.

Attempts to localize the increased phosphorylation in nuclear subfractions revealed a significant increase in both nuclear histones and acidic proteins (Fig. 4). Electrophoretic separation of the nuclear acidic proteins revealed a significant effect on the proteins in one section of the gel (Fig. 5). It is possible that the cytoplasmic steroid-receptor complex selectively enhanced the phosphorylation of this group of nuclear acidic proteins. This may result in specific interactions of these proteins and DNA leading to increased RNA synthesis.

Summary. Glucocorticoids are known to bind to liver cytosol proteins and alter nuclear RNA synthesis. Nuclear proteins are believed to assist in the regulation of nuclear RNA synthesis. The work reported here tested the possibilities that one of the very early effects of corticosterone treatment in rats consists of altered phosphorylation of nuclear proteins and that cytosol steroid-receptor complexes are necessary for altered phosphorylation of nuclear proteins.

It was found that phosphorylation of proteins in liver nuclei isolated from rats previously injected with corticosterone was significantly ($p < 0.05$) greater than control. Corticosterone *in vitro* had no effect when incubated directly with nuclei, but phosphorylation was significantly ($p < 0.05$) increased in nuclei previously incubated with rat liver cytosol containing 10^{-7} M corticosterone.

Corticosterone treatment increased phosphorylation in histones and nuclear acidic proteins. Although phosphorylation of histones was generally increased, no selective phosphorylation of any specific histone fraction was observed. In contrast, corticosterone administration resulted in a significant selective increase in the phosphorylation of a nuclear acidic protein fraction.

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