Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 142-149

EFFECT OF CYCLOHEXIMIDE ON THE INDUCTION OF TRYPTOPHAN OXYGENASE mRNA BY HYDROCORTISONE IN VIVO

Linda DeLap and Philip Feigelson, Department of Biochemistry and The Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

Received March 27,1978

SUMMARY: Hydrocortisone increases rat liver tryptophan oxygenase mRNA activity as measured by a translational assay. Pretreatment of rats with cycloheximide thirty minutes before hydrocortisone administration largely prevents the hormonal induction of tryptophan oxygenase mRNA. Tryptophan oxygenase mRNA activity begins to increase after a lag of at least 30 to 60 minutes after hydrocortisone injection. These results suggest that the synthesis of intermediary protein(s)is required for the induction of tryptophan oxygenase mRNA by glucocorticoids.

The induction of rat liver tryptophan oxygenase (tryptophan 2,3-dioxygenase, E.C. 1.13.11.11) activity by glucocorticoids occurs through an increase in the level of its functional mRNA as measured by cell-free translation (1,2). This system affords opportunities for the study of the mechanism of action of glucocorticoids. A fundamental question is whether the glucocorticoid-receptor complex is directly responsible for the induction of tryptophan oxygenase mRNA or whether the increase in this mRNA activity is a secondary effect of the hormone. Since there are currently no means of directly assaying the transcriptional rate of this gene or the processing of the gene transcript to functional mRNA, indirect experiments have been done to probe the mechanism underlying the hormonal induction of tryptophan oxygenase mRNA. The results presented in this paper indicate that there is an interval of about one hour between hydrocortisone administration and the appearance of increased levels of rat liver tryptophan oxygenase mRNA activity and that this specific mRNA induction requires ongoing protein synthesis.

MATERIALS AND METHODS

Male Sprague-Dawley rats (150-200 g) were purchased from ARS Sprague-Dawley Co. Cycloheximide was obtained from Boehringer Mannheim Biochemical Co.

0006-291X/78/0821-0142$01.00/0

Hydrocortisone (Cortef ^{R}) acetate was a gift from the Upjohn Co. Hydrocortisone phosphate was a product of Merck Pharmaceutical Co. [^{3}H] Leucine was obtained from Amersham Co. [^{3}H]Lysine and [^{14}C] orotic acid hydrate were from New England Nuclear Co.

Rats were sacrificed by cervical dislocation; the livers were removed, frozen in liquid nitrogen and stored at -80° C. Tryptophan oxygenase activity was assayed in crude liver homogenates by the method of Feigelson and Greengard (3). *In vivo* total protein and tryptophan oxygenase synthetic rates were estimated by the injection of 0.5 mCi of [^{3}H]leucine/100 g body weight 30 minutes before sacrifice of the rats. A 100,000 g supernatant was prepared as described (4) and used for the determination of incorporation of [^{3}H]leucine into total proteins and into tryptophan oxygenase (isolated by immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis) (5). These results were expressed as cpm per g of liver. RNA synthesis was estimated by injection of 10 uCi of [^{14}C]orotic acid/100 g body weight 30 minutes before sacrifice. To measure total RNA synthetic rate, samples were prepared from liver homogenates and counted as described (6). Synthesis of poly (A)-containing RNA was determined as cpm incorporated into the RNA obtained following phenol extraction (7) and poly (U) mica chromatography (8). The level of tryptophan oxygenase mRNA in poly (A)-containing RNA preparations was measured by a translational assay using the wheat germ cell-free system as described previously (9). This mRNA activity is expressed as cpm of [^{3}H]leucine and [^{3}H] lysine incorporated into newly synthesized tryptophan oxygenase (isolated by immunoprecipitation and sodium dodecyl sulfate polyacrylamide gel electrophoresis) per 10^{7} cpm incorporated into total released polypeptide chains.

RESULTS

Table 1 shows the results of an experiment to determine the effect of cycloheximide on the induction of tryptophan oxygenase mRNA by hydrocortisone. The dose of cycloheximide used caused almost complete inhibition of protein synthesis. Thus no increase in the amount of enzyme, i.e., in the catalytic activity or in the *in vivo* rate of biosynthesis of tryptophan oxygenase protein was observed after hydrocortisone administration to the cycloheximide treated animals. It is noteworthy that cycloheximide dramatically inhibited the hormonal elevation of the mRNA for tryptophan oxygenase (Table 1). Acrylamide gel patterns of the translation products of mRNAs from this experiment are shown in Figure 1. Figure 1A demonstrates that treatment with cycloheximide alone for four hours had little effect, whereas in six hours it resulted in a slight but significant decrease in the basal tryptophan oxygenase mRNA activity. Cycloheximide pretreatment largely prevented the hormonal induction of tryptophan oxygenase mRNA activity which is normally observed at four and at six hours after hydrocortisone treatment (Figures 1B and 1C). The total translational activity of poly (A)-containing RNA obtained from the livers of rats treated

Table 1

Effect of cycloheximide on the induction of tryptophan oxygenase mRNA by hydrocortisone

Treatment			In vivo biosynthesis		mRNA translation	
Cyclo-heximide	Hydro-cortisone	Catalytic activity	Total protein	Tryptophan oxygenase	Total released chains	Tryptophan oxygenase
h before sacrifice						
-	-	100	100	100	100	100
-	4	244	98	530	91	543
4.5	-	53	7	2	95	120
4.5	4	49	11	12	85	132
-	6	315	128	ND	105	1140
6.5	-	26	6	9	107	82
6.5	6	32	2	1	87	200

Where indicated rats received 10 mg/kg cycloheximide and/or 50 mg/kg hydrocortisone acetate intraperitoneally at the indicated times before sacrifice. Pooled livers from three rats were used to determine tryptophan oxygenase catalytic activity and functional mRNA level. One additional rat from each group received 1 mCi of [^{3}H] leucine 30 minutes before sacrifice enabling evaluation of *in vivo* tryptophan oxygenase and total protein biosynthetic rates. Results are expressed as per cent of control values, which were as follows: tryptophan oxygenase activity, 1.52 umoles of kynurenine/h/g of liver; *in vivo* total protein synthesis, 1.59 x 10^{6} cpm/g of liver; *in vivo* enzyme synthesis, 1220 cpm in tryptophan oxygenase/g of liver; mRNA translational activity, 9.64 x 10^{6} cpm in total released chains, 3260 cpm in tryptophan 2,3-dioxygenase/10^{7} cpm of total released polypeptide chains. ND-not done

with cycloheximide was equal to that of poly (A)-containing RNA from control and from hydrocortisone-treated rats (Tables 1 and 2).

In a parallel experiment (Table 2), cycloheximide was shown to cause only a 20-30% inhibition of [^{14}C]orotic acid incorporation into total RNA and poly (A)-containing RNA, while the hormonal induction of tryptophan oxygenase mRNA was completely prevented. Some reduction in total RNA synthesis was expected, since cycloheximide is known to cause a decrease in nucleolar RNA polymerase I activity (10,11).

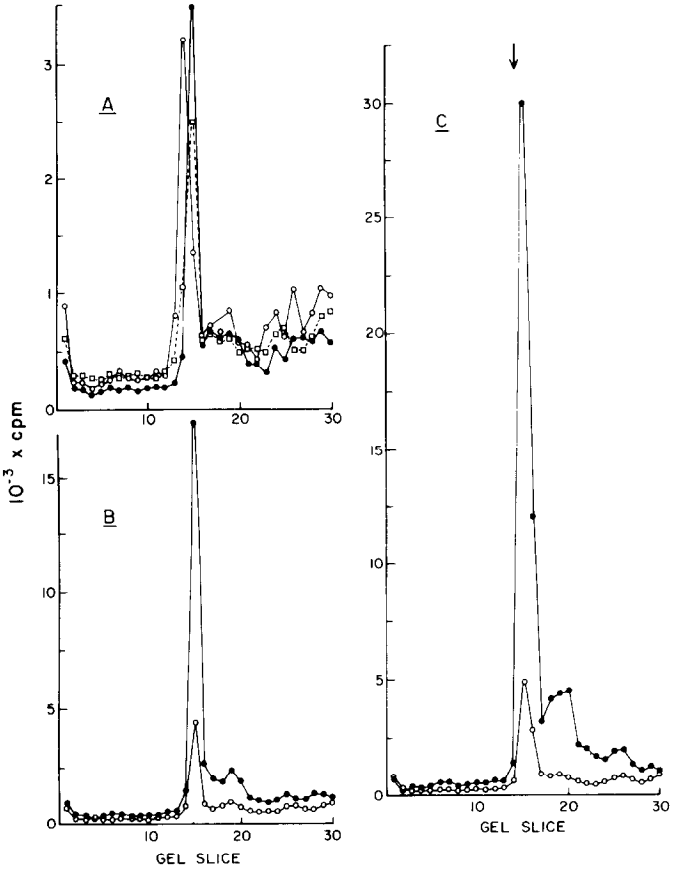

Figure 1. The inhibition by cycloheximide of glucocorticoidal induction of tryptophan oxygenase mRNA. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of immunoprecipitated translation products for the experiment shown in Table 1. Poly (A)-containing RNA was prepared from pools of three livers and translated in the wheat germ cell-free system as described. About 10^7 cpm (0.823 - 1.28 x 10^7) of released polypeptide chains were used for each immunoprecipitate. The data presented have been normalized to reflect the results expected if 1.0 x 10^7 cpm had been used for each sample. A: control rats sacrificed at zero time (●—●) and rats receiving cycloheximide and sacrificed 4.5 hours later (O—O) or 6.5 hours later (□—□); B: rats receiving hydrocortisone at 30 minutes (●—●) or cycloheximide at zero time and hydrocortisone at 30 minutes (O—O) and sacrificed at 4.5 hours; C: rats receiving hydrocortisone at 30 minutes (●—●) or cycloheximide at zero time and hydrocortisone at 30 minutes (O—O) and sacrificed at 6.5 hours. The arrow indicates the position of authentic tryptophan oxygenase.

The apparent dependence of the induction of tryptophan oxygenase mRNA upon protein synthesis suggests that some intermediate steps may occur between the intial response to glucocorticoids and the increase in the level of this mRNA.

Table 2

Effect of cycloheximide on RNA synthesis and on the induction of tryptophan oxygenase mRNA by hydrocortisone

Treatment			In vivo biosynthesis				mRNA translation	
Cyclo-heximide h before sacrifice	Hydro-cortisone	Catalytic Activity	Enzyme	Total protein	Total RNA	Total mRNA	Total released chains	Tryptophan oxygenase
-	-	100	100	100	100	100	100	100
-	6	470	700	130	109	90	119	465
6.5	-	30	2	17	68	80	108	139
6.5	6	35	23	29	116	140	72	146

Rats received 10 mg/kg cycloheximide or 50 mg/kg hydrocortisone acetate or both intraperitoneally at the indicated times before sacrifice. Pooled livers from 3 rats were used to determine tryptophan oxygenase catalytic activity, RNA synthetic rate and tryptophan oxygenase mRNA activity. Two additional rats from each group each received 0.5 mCi of [^{3}H]leucine /100 g body weight 30 minutes before sacrifice for measurement of protein and enzyme synthetic rates Results are expressed as percent of control values, which were as follows: tryptophan oxygenase activity, 3,4 umoles of kynurenine/h/g of liver; in vivo total protein synthesis, 4.59×10^{6} cpm/g of liver; in vivo tryptophan oxygenase synthesis, 4080 cpm/g of liver; RNA synthesis, 410,000 cpm/g of liver (rats received 10 uCi/100 g body weight of [^{14}C]orotic acid 30 minutes before sacrifice); mRNA synthesis, 19,000 cpm/g of liver; mRNA translational activity, 9.07×10^{6} cpm in total released chains, 1090 cpm in tryptophan oxygenase/10^{7} cpm of released polypeptide chains.

To investigate this possibility, the early time course of the hormonal induction of tryptophan oxygenase mRNA by two forms of hydrocortisone was determined (Figure 2). These results indicate a lag of at least 30 to 60 minutes before functional tryptophan oxygenase mRNA levels rise in response to the hormone.

DISCUSSION

The induction of several mRNA species by steroid hormones is characterized by a lag period (see Palmiter et al., 12). The interval between hydrocortisone administration and tryptophan oxygenase mRNA induction is longer than might be expected as a consequence of a direct interaction between the glucocor-

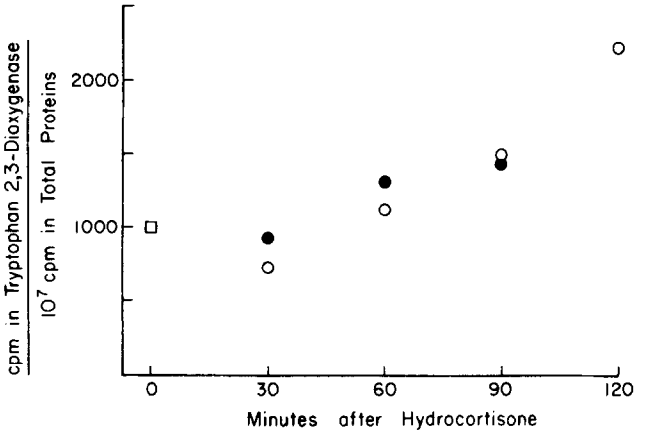

Figure 2. Time course of induction of tryptophan oxygenase mRNA by hydrocortisone. Groups of 3 male Sprague Dawley rats (160-200 g) were sacrificed at zero time (□) or received 50 mg/kg intraperitoneally of either hydrocortisone acetate (O) or hydrocortisone phosphate (●) and were sacrificed at the indicated times thereafter. Livers were pooled for preparation of poly (A)-containing RNA which was then translated in the wheat germ cell-free system. About 10^7 cpm of released polypeptide chains obtained from translation of each mRNA sample were immunoprecipitated and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The cpm in the peak comigrating with tryptophan oxygenase were taken as a measure of the tryptophan oxygenase mRNA activity. The data are normalized as in Figure 1.

ticoid-receptor complex and nuclei. At the dose employed in the present series of experiments, saturation of glucocorticoid receptors by hydrocortisone and their translocation to the nucleus is complete within 30 minutes (13). Yet, as shown here (Figure 2) no sign of elevated tryptophan oxygenase mRNA is evident until later. Yu and Feigelson (14) observed a rapid and brief increase in hepatic RNA synthesis *in vivo* after administration of cortisone to adrenalectomized rats. As early as 10 minutes after the injection of cortisone, [^{3}H]uridine incorporation increased more than 50%. The induction of mouse mammary tumor virus RNA synthesis in cell cultures by dexamethasone reaches its maximal level within 15 minutes (15) and is reported to be insensitive to protein synthesis inhibitors (16). In contrast, induction of ovalbumin mRNA in chick oviduct by estrogen begins after a lag period of about three hours and is substantially reduced by protein synthesis inhibitors (12). Similarly, the present results suggest that induction of tryptophan oxygenase mRNA activity by gluco-

corticoids requires protein synthesis. These findings are reminiscent of studies on ecdysone-induced chromosomal puffing in Chironomus (17) and Drosophila (18). The formation of early chromosomal puffs is unaffected by protein synthesis inhibitors which do prevent the induction of later puffs. Recent findings from this laboratory show that the action of dexamethasone in the induction of tyrosine aminotransferase by dexamethasone plus glucagon takes place during a two hour lag period during which protein synthesis is required (19). Surprisingly, after administration of cycloheximide, puromycin or emetine in vivo there is a several-fold increase in hepatic tyrosine aminotransferase mRNA activity. No such effect is seen for tryptophan oxygenase mRNA activity (20).

The present results, i.e., the lag period and a requirement for ongoing protein synthesis, indicate that induction of tryptophan oxygenase mRNA by glucocorticoids is a complex process which seems to be mediated by one or more short-lived proteins. These findings may be analagous to our earlier demonstration that the glucocorticoidal increase in nuclear and nucleolar transcriptive synthesis of ribosomal RNA by RNA polymerase I was prevented by prior in vivo administration of either the RNA polymerase II inhibitor alpha amanitin or cycloheximide (21). All these studies are compatible with the concept that glucocorticoidal interaction with certain genomic loci may directly induce mRNA and protein species and that they in turn may accelerate other transcriptive events including ribosomal RNA and tryptophan oxygenase mRNA synthesis.

ACKNOWLEDGEMENTS: We wish to acknowledge the skillful technical assistance of Julie Kwang. This research was supported by National Institutes of Health Grant No. CA 22376.

REFERENCES

1. Schutz, G., Killewich, L., Chen, G. and Feigelson, P. (1975) Proc. Nat. Acad. Sci. USA 72, 1017-1020.
2. Feigelson, P., Ramanarayanan-Murthy, L., and Colman, P.D. (1978) in "Receptors and Hormone Action," Eds. B.W. O'Malley and L. Birnbaumer, pp. 225-249, Academic Press, New York.
3. Feigelson, P. and Greengard, O. (1962) J. Biol. Chem. 237, 3714-3717.
4. Sippel, A.E., Kurtz, D.T., Morris, H.P., and Feigelson, P. (1976) Cancer Res. 36, 3588-3593.
5. Bollum, F.J. (1968) Methods in Enzymol. 12, 169-173.
6. Yu, F.-L., and Feigelson, P. (1971) Analyt. Biochem. 39, 319-321.

7. Schutz, G., Beato, M., Feigelson, P. (1973) Proc. Nat. Acad. Sci. USA 70, 1218-1221.
8. Pulkrabek, P., Klier, K., Grunberger, D. (1975) Analyt. Biochem. 68, 26-35.
9. Killewich, L., and Feigelson, P. (1977) Proc. Nat. Acad. Sci. USA, 74, 5392-5396.
10. Yu, F.-L., and Feigelson, P. (1972) Proc. Nat. Acad. Sci. USA 69, 2833-2837.
11. Lampert, A., and Feigelson, P. (1974) Biochem. Biophys. Res. Commun. 58, 1030-1038.
12. Palmiter, R.D., Moore, P.B., Mulvihill, E.R., and Emtage, S. (1976) Cell 8, 557-572.
13. Beato, M., Kalimi, M., and Feigelson, P. (1972) Biochem. Biophys. Res. Commun. 47, 1464-1472.
14. Yu, F.-L., and Feigelson, P. (1969) Biochem. Biophys. Res. Commun. 35, 499-506.
15. Ringold, G.M., Yamamoto, K.R., Bishop, J.M., and Varmus, H.E., (1977) Proc. Nat. Acad. Sci. USA 74, 2879-2883.
16. Ringold, G.M., Yamamoto, K.R., Tomkins, G.M., Bishop, J.M., Varmus, H.E. (1975) Cell 6, 299-305.
17. Clever, U. and Romball, C.G., (1966) Proc. Nat. Acad. Sci. USA, 56, 1470-1476.
18. Ashburner, M., Chihara, C., Meltzer, P. and Richards, G., (1973) Cold Spring Harbor Symp. Quant. Biol. 38, 655-662.
19. Ernest, M.J., Chen, C.-L. and Feigelson, P. (1977) J. Biol. Chem. 252, 6783-6791.
20. Ernest, M.J., DeLap, L., and Feigelson, P. (1978) J. Biol. Chem. 253, 2201-2203.
21. Yu, F.-L. and Feigelson, P. (1973) Biochem. Biophys. Res. Commun. 53, 754-760.