Vol. 88, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1979 Pages 189-198

CHANGES IN THE INSULIN AND GLUCAGON RECEPTORS IN THE REGENERATING LIVER FOLLOWING INTOXICATION WITH CARBON TETRACHLORIDE

Marisabel Mourelle* and Boanerges Rubalcava+

Biochemistry Department, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, P.O. Box 14-740

México 14, D. F.

Received April 2, 1979

The response of rat liver plasma membrane adenylate cyclase was studied from one to 14 days after a single dose of carbon tetrachloride (CCl_4). The response to glucagon was diminished to a greater extent than that of fluoride and was due to a deficiency in hormone binding. In contrast, insulin binding increased 300% over control; the change was due to increased number of binding sites. The "affinities" of receptors for either hormone were not altered. The tissue levels of adenosine 3':5'-monophosphate increased following CCl_4 poisoning reaching a peak precisely when the adenylate cyclase response to glucagon was at its lowest level. These studies present evidence that receptors for pancreatic hormones change differently when liver is damaged and during its regeneration following CCl_4 intoxication. The change in pattern is remarkably similar to changes reported previously in fetal liver development or following partial hepatectomy of adult rat.

It has been proposed that hepatocyte proliferation is regulated by blood-borne factors (1). In vivo evidence suggests that insulin and glucagon may be involved in this process (2-6). We have concluded (7) that liver participates in the regulation of blood levels of these hormones and that, after partial hepatectomy, hepatic regeneration appears to be accompanied by specific alterations in the membrane receptors for pancreatic hormones, namely, an increased number of insulin receptors while glucagon receptors decreased in a reversible fashion (8). We also reported (9) that the

* Presented as partial fullfilment for M.Sc. degree at Department of Pharmacology of Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional.

+ To whom correspondence and requests for reprints should be addressed.

0006-291X/79/090189-10$01.00/0

fetal liver is less sensitive to glucagon action than the adult liver, and that this glucagon "resistance" is due to a reduced capacity of the hepatocyte to bind glucagon; this period of glucagon hyposensitivity corresponds in general with the period of maximal liver growth. A reduced adenylate cyclase response to glucagon was detected but the response to sodium fluoride remained the same throughout fetal and adult life. These findings suggested a deficit in glucagon action rather than a change in the levels of adenylate cyclase. Wicks (10) has reported a relatively large adenylate cyclase response to catecholamines in the fetal liver, which can be related with the presence of functional beta adrenergic receptors.

It is well known that the exposure of animals to carbon tetrachloride (CCl_4) produces a fatty liver and hepatocellular necrosis (11). Leevy et al. (12) demonstrated that following liver toxic injury, synthesis of deoxyribonucleic acid occurs, indicating new cell formation. The acute CCl_4 intoxication of the rat causes a marked rise in serum α-fetoprotein concentration which is higher than that caused by partial hepatectomy (13); the release of this polypeptide is associated with hepatic proliferation (13, 14).

The studies reported here were designed to determine if the pattern of changes in the pancreatic hormones receptors previously seen in fetal and proliferating livers also occurs in hepatic proliferation following CCl_4 intoxication.

MATERIALS AND METHODS

Glucagon and insulin were gifts from Lilly; [α-^{32}P] ATP and cyclic AMP were supplied by the International Chemical and Nuclear Corporation. [^{3}H]cyclic AMP and [^{125}I] -iodoinsulin were purchased from New England Nuclear; creatine phosphate, creatine phosphokinase and bovine serum albumin from Sigma Chemical Co. Oxoid filters were obtained from Amersham Searle (Chicago) and [^{125}I] iodoglucagon from Nuclear Medical Laboratories (Dallas). All other reagents were of analytical grade.

Wistar male rats wighing 180-250 g fed ad libitum a Purina Chow were used for these studies. The treated animals received a single dose of 0.5 ml per 100 g body weight of CCl_4 in corn oil (1:1 v/v) through an intragastric tube; the controls received the correspondent volume of corn oil. The rats were sacrificed at different periods (see results) to prepare both liver homogenates and partially purified plasma membranes; the homogenates were prepared using 50 mM Tris-HCl, pH 7.5 with 4 mM EDTA using fresh livers. The beaker with the homogenate was transferred to a bath of boiling water for 3 minutes and then centrifuged at 15,000 x g for 30 minutes; the final supernatant was frozen and stored at -80°C and used afterwards to measure the amount of cyclic AMP according to Pollard and Weingrand (15). Partially purified plasma membranes from the rat liver were prepared according to Neville's procedure (16) with a modification described by Pohl et al (17). The membranes were frozen and stored in liquid nitrogen and, for further use, were suspended in 20 mM Tris- HCl, pH 7.5.

Adenylate cyclase activity was measured by the method described by Salomon et al (18); the assay medium contained in 0.1 ml 1 mM EDTA, 3.2 mM [α-^{32}P] ATP (20-40 cpm/pmole), 5 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.5, 1 mM cyclic AMP and an ATP regenerating system consisting of 20 mM creatine phosphate and 100 U/ml creatine phosphokinase. Either glucagon (1 µM.) or sodium fluoride (10 mM) was added to stimulate enzyme activity; incubations were carried out for 10 minutes at 30°C; the reaction was initiated by the addition of the membranes to give 30-40 µg of protein.

The binding of [^{125}I] iodoinsulin or [^{125}I] iodoglucagon to liver membranes was carried out as described by Rubalcava and Rodbell (19); 25 mm diameter oxoid filters and 2-10 day old monoiodinated [^{125}I] insulin (sp. activity 90µCi/µg) or glucagon (sp. activity 400 µCi/µg) were used. Greater than 90% of radioactivity associated with each probe was precipitable with trichloroacetic acid. Nonspecific binding (radioactivity retained by filters from parallel assay tubes incubated together with 10µM unlabelled hormone) was subtracted to obtain "specific binding". Liver membranes (30-50 µg of protein) were incubated at 30°C in a final volumen of 0.1 ml containing 20 mM Tris-HCl, pH 7.5, 1% bovine serum albumin and the radioactive hormone at different concentrations. Incubation terms were 20 minutes for glucagon binding and 30 minutes for insulin. Protein was measured by the procedure of Lowry et al (20) using bovine serum albumin as standard.

RESULTS

Fig. 1 illustrates the effect of CCl_4 on the response of adenylate cyclase to glucagon and fluoride at different periods after intoxication. One day after the administration of the toxic agent the basal activity (non-stimulated) as well as those stimulated either by glucagon or fluoride were diminished; at the third day all activities recovered to the control values. Fluoride ion stimulates adenylate cyclase by a different process from

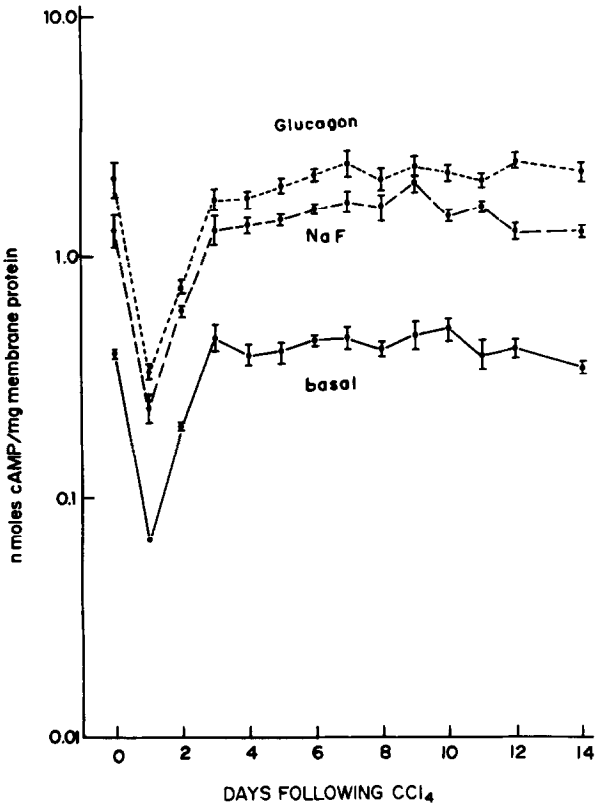

Fig. 1: Effect of CCl_4 on response of adenylate cyclase to glucagon (1 μM) and fluoride ion (10 mM). Incubation conditions and procedures for assaying adenylate cyclase activity are described under methods. Each point represents the mean of 25 experiments ± S.D.

hormonal activation (21). The response of the enzyme to this ion is useful, therefore, for distinguishing the possible effects of CCl_4 on receptor-mediated versus the process by which fluoride acts. It has been postulated that CCl_4 affects the interaction of lipids with the carrier protein in the transport of the lipids (11) and we showed that acidic phospholipids are required for glucagon but not for fluoride to affect the rat liver adenylate cyclase (19); therefore the data of Fig. 1 were calculated as the ratio of the different activities: fluoride/basal, glucagon/basal and glucagon/fluoride. The ratio fluoride/basal remained constant (Table I), while the glucagon/basal and glucagon/fluoride ratios

TABLE I

ADENYLATE CYCLASE ACTIVITY RATIOS IN PLASMA MEMBRANES FROM LIVERS OF RATS INTOXICATED WITH CCl_4

DAYS FOLLOWING CCl_4	FLUORIDE / BASAL	GLUCAGON / BASAL	GLUCAGON / FLUORIDE
0	3.35*	5.43	1.61
1	3.56	2.42	0.96
2	2.96	2.54	0.86
3	2.93	3.83	1.30
4	3.52	4.48	1.27
5	3.52	4.96	1.41
6-14	similar to the last value	similar to the last value	similar to the last value

* The ratios were calculated from the data plotted in Fig. 1, and represent the mean of 25 experiments.

fell 24 and 48 hours after the intoxication and returned to the control values 72 hours later. Since the ratio fluoride/basal remains unchanged it seems that the response due to glucagon is more affected than the others; we measured the amount of cyclic AMP in the supernatant prepared from the homogenates of livers during the same periods (Fig. 2). The Levels of the nucleotide did not change in 24 hours but then increased to a peak in 48 hours; this peak occurred precisely when the adenylate cyclase response to glucagon was at its lowest level. This finding is further evidence that glucagon action changes differently owing to CCl_4 intoxication.

To determine if the loss of response of the adenylate cyclase to glucagon was the consequence of a difference in the binding of glucagon to its receptors, the binding of $[^{125}I]$ iodoglucagon

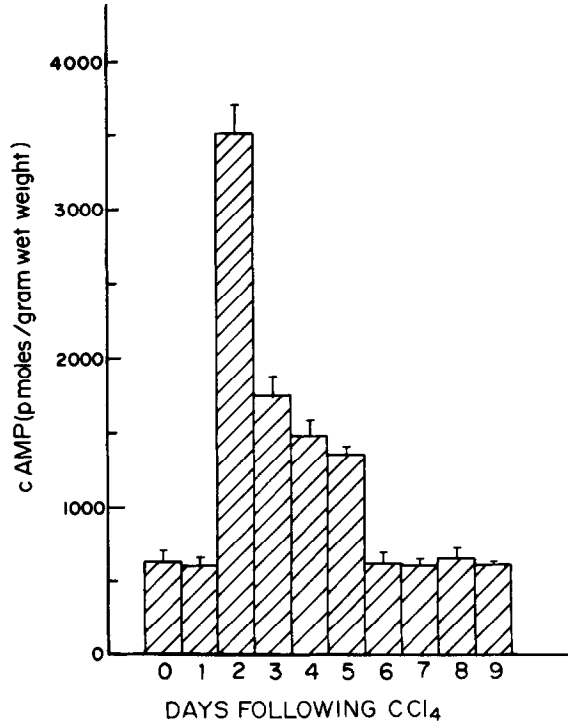

Fig. 2: Effect of CCl_4 on the levels of cAMP determined in rat liver homogenates. Each bar represents the mean of 20 experiments ± S.D. The procedure for assaying cAMP is described under methods.

to liver membranes was compared at various time points after the intoxication with CCl_4 (Fig. 3 and Table II). Glucagon binding decresed sharply in the first day after the administration of the toxic agent (from 3.73 to 0.63 pmoles/mg of protein) and remained at the low value on the second day; finally, as is the case of fluoride-stimulated adenylate cyclase activity, it returned to control values on the third day. On the other hand, during the first two days there were no changes in the binding of insulin (the small fall occurring at the first day and the increase seen the second day are not significant). However, on the third day, binding of insulin increased to nearly 300% of the control value; thereafter the binding returned gradually to the initial value. The apparent affinity values of the peptides

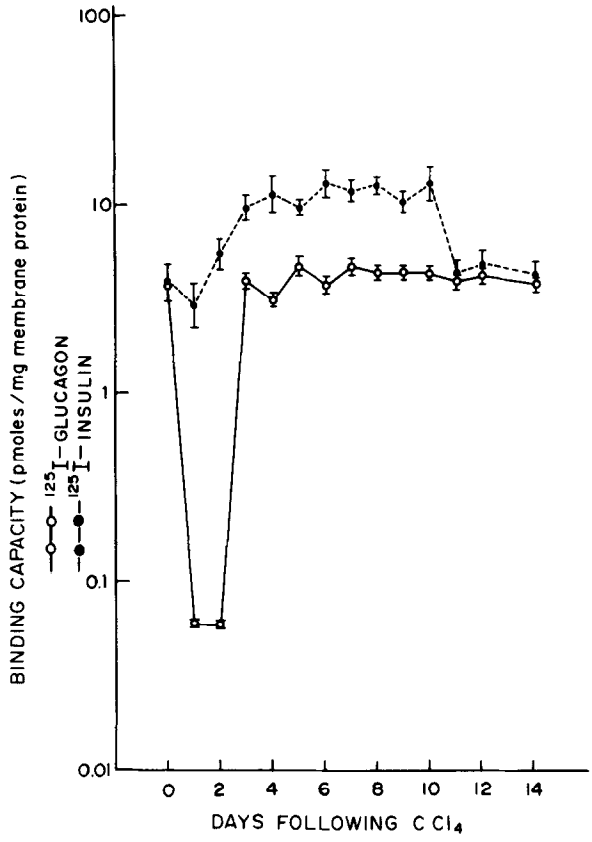

Fig. 3: Effect of CCl_4 on the binding of [^{125}I] iodoinsulin and [^{125}I] iodoglucagon. Incubation conditions and procedures for assaying binding of labeled glucagon and insulin are described under methods. The results are expressed as mean value of 25 experiments ± S.D.

for their receptors, obtained by Scatchard plots (22), did not change (Table II). We were unable to detect high affinity sites for insulin.

DISCUSSION

The effect of adenylate cyclase activity following CCl_4 administration was a different one for the activity stimulated by glucagon and the basal and fluoride stimulated activities (Table I). Thus the effect of CCl_4 on hormone action seems to be greater than that on the catalytic component of the adenylate cyclase system. In fact, the rise in the tissue levels of cyclic AMP during the fall

TABLE II

EFFECT OF CCl_4 ON BINDING OF $[^{125}I]$ GLUCAGON AND $[^{125}I]$ INSULIN TO RAT LIVER PLASMA MEMBRANES

DAYS FOLLOWING CCl_4	GLUCAGON		INSULIN	
	$K_a \times 10^8$ M^{-1}	BINDING CAPACITY (pmol/mg)	$K_a \times 10^7$ M^{-1}	BINDING CAPACITY (pmol/mg)
0	1.38 ± 0.11*	3.73 ± 0.64	1.25 ± 0.05	3.85 ± 1.07
1	1.26 ± 0.15	0.06 ± 0.00	1.16 ± 0.15	2.92 ± 0.95
2	1.10 ± 0.13	0.06 ± 0.00	1.11 ± 0.20	5.55 ± 1.66
3	1.46 ± 0.10	3.96 ± 0.21	0.99 ± 0.07	9.89 ± 1.27
4	1.50 ± 0.12	3.16 ± 0.32	0.92 ± 0.06	10.12 ± 2.26
5	1.41 ± 0.05	4.69 ± 0.71	1.12 ± 0.13	0.57 ± 1.27
6	1.38 ± 0.10	3.62 ± 0.36	1.06 ± 0.20	12.63 ± 1.40
7	1.54 ± 0.13	4.63 ± 0.51	0.90 ± 0.11	11.64 ± 2.20
8	1.11 ± 0.20	4.39 ± 0.42	0.96 ± 0.04	12.67 ± 1.40
9	1.16 ± 0.15	4.40 ± 0.34	0.95 ± 0.17	10.30 ± 1.35
10	1.43 ± 0.14	3.88 ± 0.25	1.00 ± 0.20	13.00 ± 3.00
11	1.07 ± 0.26	3.92 ± 0.32	1.10 ± 0.10	4.20 ± 0.70
12	1.52 ± 0.12	4.21 ± 0.54	1.14 ± 0.10	4.80 ± 1.05
14	1.36 ± 0.11	3.77 ± 0.43	1.41 ± 0.13	4.30 ± 0.98

*The binding capacity and K_a were obtained from Scatchard plots. Each value represents the mean value of 25 experiments ± S.D. Incubation conditions and procedures for assaying binding of labeled hormones are described under methods concentrations were for either hormone from 0.5 nM to 1 μM.

in glucagon response suggests that the enzyme is minimally altered by CCl_4 treatment. The high levels of the nucleotide could be explained by the high plasma levels of catecholamines after the intoxication (23). An alternative possibility is inactivation of phosphodiesterase enzymes.

Since the receptors for $[^{125}I]$ iodoinsulin were not damaged while the $[^{125}I]$ iodoglucagon receptors were reduced markedly, it appears that the effects of CCl_4 are selective for the glucagon receptor. Thus, CCl_4 induces the same pattern of receptor changes observed in fetal proliferation liver of following hepatoctomy in regenerating adult liver.

We are in the process of examining if these alterations involve synthesis and degradation or masking and unmasking of the peptide receptors. In the case of insulin, it is interesting that the apparent increase in number of receptors seen either in the regenerating liver following CCl_4 or partial hepatectomy coincides in time with the appearance of α-fetoprotein. The synthesis of this protein following CCl_4 is suppresed with mitomycin C (13) which has been reported to inhibit the synthesis of RNA in addition to DNA (24). Experiments are in progress using this antibiotic to examine the effect of CCl_4 on the number of insulin receptors under the same conditions reported here.

Acknowledgments. We are indebted to Miss Eunice Zavala and Mr. Agustín Martínez for superb technical assistance.

REFERENCES

1. Fisher, B., Szuch, P. and Fischer, E.R. (1971) Cancer Res. 31, 322-331
2. Rabes, H.M. and Brandle, H. (1969) Cancer Res. 29, 817-822
3. Echave Llanos, J.M. Gomez Dumm, C.L. and Surur, J.M. (1971) Experientia 27, 574-575
4. Rixon, R.H. and Whifield, J.F. (1972) Proc. Soc. Exp. Biol. Med. 141, 93-97

5. Leffert, H. (1974) J. Cell Biol. 62, 792-801
6. Bucher, N.L.R. and Seaffield, M.N. (1975) Proc. Nat. Acad. Sci. USA 72, 1157-1160
7. Leffert, H., Alexander, N.M., Faloona, G., Rubalcava, B. and Unger, R. (1975) Proc. Nat. Acad. Sci. USA 72, 4033-4036
8. Leffert, H.L., Koch, K.S. Rubalcava, B., Sell, S., Moran, T. and Boorstein, R. (1978) J. Natl. Cancer Inst. Monogr. 48 87-101
9. Blazquez, E., Rubalcava, B., Montesano, R., Orci, L. and Unger, R. (1976) Endocrinology 98, 1014-1024
10. Wicks, J. (1969) J. Biol. Chem. 244, 3941-3950
11. Recknagel, R.O. (1967) Pharmacol. Rev. 19, 145-208
12. Leevy, C.M., Hollister, R.M., Schid, R., MacDonald, R.A. and Davidson, C.S. (1959) Proc. Soc. Exp. Biol. and Med. 102, 672-675
13. Taketa, K., Watanabe, A. and Kosaka, K. (1975) Ann, N.Y. Acad. Sci. 259, 80-89
14. Smuckler, E.A., Kiplitz, M. and Sell, S. (1976) Cancer Res. 36, 4558-4561
15. Pollard, H.B. and Weingrand, D. (1976) Anal. Biochem. 76, 382-384
16. Neville, D.R. (1968) Biochem. Biophys. Acta 154, 540-552
17. Pohl, S.L., Birnbaumer, L. and Rodbell, M. (1971) J. Biol. Chem. 246, 1849-1856
18. Salomon, Y., Londos, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541-548
19. Rubalcava, B., and Rodbell M. (1973) J. Biol. Chem. 248, 3831-3837
20. Lowry, O.H., Rosenbrough, N.Y., Farr, A.L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
21. Rodbell, M. (1972) in Glucagon: Molecular Physiology, Clinical and Therapeutic Implications (Lefevbre, P.S. and Unger, R.H., eds.) pp. 61-75 Pergamon Press, New York.
22. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51: 660-675
23. Stern, P.H., and Brody, T.M. (1963) J. Pharmacol. Exp. Ther. 141, 65-73
24. McCann, J.J.T., Lo, M. and Webster, D.A. (1971) Cancer Res. 31, 1573-1579.