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DECREASED cAMP RESPONSIVENESS TO GLUCAGON IN LIVERS FROM ETHYNYL ESTRADIOL TREATED RATS [1]

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Summary

The effects of glucagon on the concentration and output of cAMP were studied in liver slices and in perfused livers from female rats and from animals treated with ethynyl estradiol (15 µg/kg daily for 14 days). The basal content of cAMP in liver slices, or of cAMP released into the perfusion medium in the absence of glucagon, was unaffected by prior treatment of the animal with estrogen. When glucagon was added to the medium, the concentration of cAMP in liver slices was 2.29 ± 0.32 and 1.10 ± 0.11 pmol cAMP/mg wet weight from control and ethynyl estradiol treated rats, respectively. When glucagon was added, the output of cAMP by perfused livers was maximal at 20 minutes with livers from either control or ethynyl estradiol treated rats. Output of cAMP by the perfused liver, when glucagon was added to the medium, was 8.76 ± 0.69 and 1.84 ± 0.08 nmol/g by livers from control and ethynyl estradiol treated rats, respectively. This effect was the same whether animals had been fasted for 12 hours previously, or were allowed free access to food until sacrifice. Clearly, as measured by cAMP accumulation, prior treatment of the rat with ethynyl estradiol reduced the sensitivity of the hepatic cAMP response to glucagon

Introduction

The addition of glucagon or dibutyryl cAMP[3] to the media perfusing livers from fed male rats reduced the output of triglyceride and stimulated ketogenesis (1,2). In contrast, glucagon, at a concentration of 0.1 µM, did not inhibit the output of triglyceride by livers from fed female rats (3), even though this same concentration of glucagon inhibited triglyceride output by livers from

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3. Abbreviations: cAMP, cyclic adenosine 3'5' monophosphate; dibutyryl cAMP, N^6-O^2-dibutyryl cyclic-adenosine 3'5' monophosphate; MIX, methylisobutylxanthine.

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male animals under similar experimental conditions (1). Treatment of the animal *in vivo* with glucagon was also reported by McGarry and colleagues to stimulate ketogenesis by the isolated perfused liver from male rats, although these workers were unable to demonstrate an action of the hormone when added to the medium *in vitro* (4). Glucagon, moreover, lowers blood triglyceride levels (5), particularly in the insulin deficient state (6). In contrast to the catabolic actions of glucagon on hepatic lipid metabolism are the anabolic effects of estrogens. Treatment of female rats for 14 days with ethynyl estradiol produced hypertriglyceridemia (7) and stimulated the output of triglyceride by the isolated perfused liver (8,9). The incorporation of [1-^{14}C] oleate into triglyceride by livers from ethynyl estradiol treated female rats was increased, while incorporation into ketone bodies was reduced, in comparison to the controls (9). These actions of the steroid hormone are opposite to those observed with glucagon. These observations prompted the thought that these chemically dissimilar hormones might act on hepatic metabolism by differentially affecting some final common pathway. Estrogen treatment *in vivo* might even modify the response of the liver to glucagon. Since the polypeptide hormone glucagon is thought to act, at least in part, through stimulation of the adenylate cyclase system, the effects of pretreatment *in vivo* with ethynyl estradiol on the production of cAMP in response to glucagon was measured in isolated perfused livers and in liver slices from control and estrogen-treated female rats.

Materials and Methods

Female rats (175-225 g) of the Sprague Dawley strain were obtained from the Charles River Breeding Laboratories, Wilmington, MA and were maintained on 15 g/day Purina laboratory chow for 14 days (8) and water *ad libitum*. Food was removed on the last day 10-12 h before the animals were killed. In one experiment, rats were fed *ad libitum* 48 h prior to removal of the livers for perfusion. One group of rats received 15 µg ethynyl estradiol/kg in sesame oil daily by subcutaneous injection, and one group received vehicle alone during the 14 day experimental period.

Rats were killed by decapitation, and slices (0.5 mm) were prepared from the livers of control and estrogen treated rats using a Stadie-Riggs microtome. Slices from each liver were used for parallel experiments with and without 1.0 µM glucagon. Each flask contained 40-60 mg of slices in 2 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4 containing 10 mM glucose, 0.25 g/dl bovine serum albumin and 1 mM MIX. The slices were preincubated in this medium for

15 minutes; glucagon or vehicle was then added and the incubation was continued for an additional 10 minutes. The incubation was stopped by transferring the slices to 0.5 ml acetate buffer, pH 6.2, and heating at 100°C for 3 minutes.

Rats from control and treatment groups were selected at random and anesthetized lightly with diethyl ether. The livers were removed surgically (10) and perfused with media (9) and in an apparatus described previously (11). After a 20 minute equilibration period, oleic acid (2.25 μmol/min) was infused without glucagon for 30 minutes, after which oleic acid with glucagon was infused for 60 minutes. During this 60 minute period, 135 μmol oleic acid and 44.6 μg glucagon were infused. After 60 minutes infusion of glucagon, the final concentration would be 1.5×10^{-7} M, assuming no catabolism of the hormone by the liver. Since approximately 80% of the biologically active glucagon may be extracted by the liver (12), the effective concentration of glucagon was probably considerably lower than calculated for these experiments.

Samples of perfusate were removed before and after administration of glucagon for determination of concentration of cAMP. cAMP was extracted from liver slices and perfusion medium with 0.3 N perchloric acid. The neutralized extracts were acetylated (13) and cAMP was determined by radioimmunoassay (14). Chemicals and solvents of reagent grade were obtained from Fisher Scientific Corp., St. Louis, MO and from Sigma, Inc., St. Louis, MO. Glucagon was the gift of Dr. Erold Diller, Eli Lilly and Co., Indianapolis, IN. Ethynyl estradiol was the gift of Dr. Enrico Forchielli, Syntex Corp., Palo Alto, CA.

Results and Discussion

Initial experiments were carried out with liver slices incubated in the presence of the cyclic nucleotide phosphodiesterase inhibitor, MIX. The basal content of cAMP in liver slices from control and estrogen-treated female rats was similar in the absence of glucagon (Fig. 1). Stimulation of the adenylate cyclase system by 1 μM glucagon however, as measured by the subsequent increase in cAMP concentration in the slices was reduced in the estrogen-treated group. The cAMP response of liver slices from estrogen-treated rats was about 50% of the control response. Since the liver slices were incubated in the presence of 1 mM MIX, the refractory cAMP response to glucagon in slices from estrogen-treated rats may be due to decreased formation of cAMP rather than to increased catabolism of cAMP by phosphodiesterase. This, however, remains to be determined. It is of related interest that an increase in arterial phosphodiesterase activity has been reported in estrogen treated rabbits (15).

The cAMP response of the isolated perfused rat liver to glucagon was also examined. Infusion of glucagon did not affect the uptake of free fatty acid by the liver (1). The appearance of cAMP in the perfusate was measured before

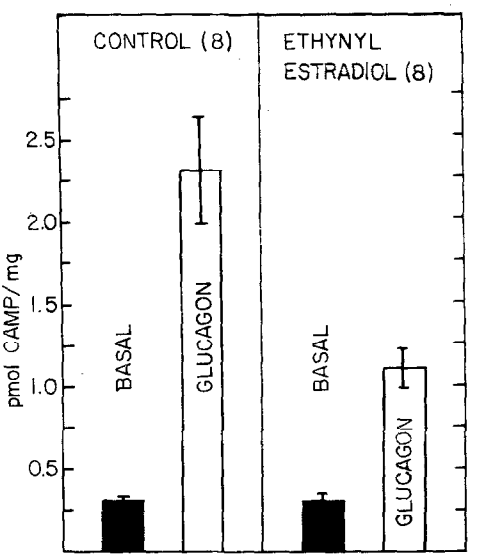

Figure 1. Effects of glucagon on the concentration of cAMP in liver slices from control and ethynyl estradiol treated rats. See text for details of incubations. Values are means ± SE. Numbers of experiments are shown in parentheses.

Significance of differences:

1. Basal vs glucagon (control), $p < 0.001$
2. Basal vs glucagon (ethynyl estradiol), $p < 0.001$
3. Basal (ethynyl estradiol) vs basal (control), not significant
4. Glucagon (ethynyl estradiol) vs glucagon (control), $p < 0.01$

and during infusion of glucagon into the medium. This measurement was considered to reflect the response of the tissue cAMP to the hormone (16), although the relative efflux of cAMP from livers of control and estrogen-treated rats must be considered in the interpretation of these data. Figure 2 depicts the appearance of cAMP in the perfusate before and during infusion of glucagon into the medium. The output of cAMP into the perfusate was similar in experiments with livers from control and estrogen-treated animals in the absence of glucagon. The net output of cAMP into the medium in response to glucagon was depressed in livers from estrogen-treated animals relative to the control group. This refractory cAMP response to the peptide hormone was observed whether the animals were fed ad libitum for 48 hours prior to sacrifice or were fasted for the last 12 hours before the animals were killed. This consideration was

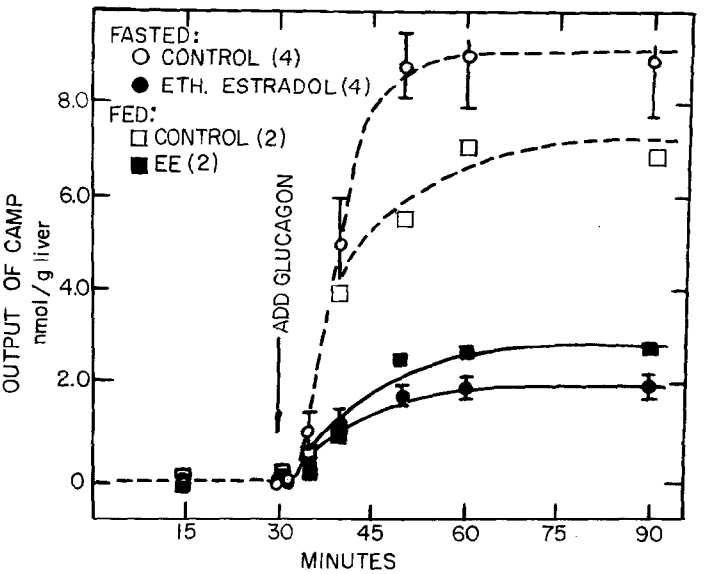

Figure 2. Effect of treatment with ethynyl estradiol on the cAMP response to glucagon by liver. Data were obtained with livers from fasted (10-12 hr) or ad libitum fed (48 hr.) female control and estrogen treated rats. See text for details of perfusions. Values are means ± SE. Numbers of perfusions are shown in parentheses. Ten minutes after the start of the infusion of glucagon, and at all subsequent intervals, the output of cAMP into the perfusate was depressed by pretreatment with estrogen, $p < 0.001$.

important since the nutritional state of the animal may alter the cAMP response of the liver to glucagon (17). The experiments were conducted in the absence of a phosphodiesterase inhibitor, in contrast to the experiments with liver slices. Clearly the estrogen-glucagon interaction was readily demonstrable whether or not the hepatic phosphodiesterase system was inhibited.

These studies have demonstrated that treatment of the rat with the estrogen, ethynyl estradiol, reduced dramatically the response of liver to glucagon as measured by cAMP accumulation. This interaction between glucagon and estrogen may have important physiologic consequences relative to the effects of estrogen on hepatic lipid and carbohydrate metabolism. Since glucagon has important regulatory actions on hepatic lipid and carbohydrate metabolism which may be mediated by cyclic nucleotides (1,2,18), the interaction between the steroid hormone, estrogen and the peptide hormone,

glucagon to alter the cAMP response is of particular interest. Whether the altered cAMP response to glucagon in livers from estrogen-treated rats is the result of increased rates of cAMP catabolism (increased phosphodiesterase activity) or decreased cAMP synthesis (decreased adenylate cyclase activity) remains to be determined. The effects of the estrogen on the cAMP response were clearly demonstrable when the phosphodiesterase inhibitor, MIX, was used in the experiments with liver slices. The direction of future work will be to delineate relationships between the altered cAMP response to glucagon, induced by pretreatment with the estrogen, and hepatic lipid metabolism.

References

1. Heimberg, M., Weinstein, I., and Kohout, M. (1969) J. Biol. Chem. 244, 5131-5139.
2. Klausner, H.A., Soler-Argilaga, C., and Heimberg, M. (1978) Metabolism 27, 13-25.
3. Weinstein, I., Seltzer, M., and Belitsky, R. (1974) Biochim. Biophys. Acta 348, 14-22.
4. McGarry, J.D., Wright, P.H., and Foster, D.W. (1975) J. Clin. Invest. 55, 1202-1204.
5. Eaton, R.P. (1973) J. Lipid Res. 14, 312-317.
6. Schade, D.S., and Eaton, R.P. (1975) Diabetes 24, 510-515.
7. Weinstein, I., Turner, F.C., Soler-Argilaga, C., and Heimberg, M. (1978) Biochim. Biophys. Acta 530, 394-401.
8. Weinstein, I., Seedman, S., and Veldhuis, M. (1975) Proc. Soc. Exp. Biol. Med. 149, 181-184.
9. Weinstein, I., Soler-Argilaga, C., and Heimberg, M. (1977) Biochem. Pharmacol 26, 77-80.
10. Kohout, M., Kohoutova, B., and Heimberg, M. (1971) J. Biol. Chem. 246, 5067-5074.
11. Heimberg, M., Fizette, N.B., and Klausner, H.A. (1964) J. Amer. Oil Chem. Soc. 41, 774-779.
12. Jaspan, J.B., Huen, A. H-J., Morley, C.G., Moossa, A.R., and Rubinstein, A.H. (1977) J. Clin. Invest. 60, 421-428.
13. Harper, J.F., and Brooker, G. (1975) J. Cyclic Nuc. Res. 1, 207-218.
14. Steiner, A.L., Kipnis, D.M., Utiger, R., and Parker, C. (1969) Proc. Natl. Acad. Sci. USA 64, 367-373.
15. Numano, F., Maezowa, H., and Shimamoto, T. (1977) Third Int'l. Cong. Cyclic Nucleotides 69.
16. Exton, J.H., Robison, G.A., Sutherland, E.R., and Park, C.R. (1971) J. Biol. Chem. 246, 6166-6177.
17. Broer, Y., Freychet, P., and Rosselin, G. (1977) Endocrinology 101, 236-249.
18. Exton, J.H., Lewis, S.B., Ho. R.J., and Park, C.R. (1972) Adv. Cyclic Nuc. Res. 1, 91-101.