EFFECTS OF HUMAN CHORIONIC GONADOTROPIN AND N^6-$O^{2'}$-DIBUTYRYL ADENOSINE-3',5'-MONOPHOSPHATE ON PHOSPHOFRUCTOKINASE ACTIVITY IN ISOLATED RAT OVARIES

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Received March 16,1976

SUMMARY

Phosphofructokinase activity in prepubertal rat ovaries is elevated by *in vitro* treatment with human chorionic gonadotropin or N^6-$O^{2'}$-dibutyryl-adenosine-3',5'-monophosphate. Puromycin and actinomycin D have no appreciable effect on the hormone-induced enzyme increase but the stimulatory effect of the cyclic nucleotide is blocked by puromycin. In the light of these and other observations, the possible mode of action of the gonadotropin is discussed.

The stimulatory action of HCG* on the carbohydrate metabolism in its target organs has been well established. Demers *et al.* (1) demonstrated that HCG elicits a glycogenolytic response in human placenta. Earlier, Hamberger and Ahren (2) showed that glucose uptake and lactic acid production in the isolated rat ovary are markedly stimulated by the addition of HCG to the incubation medium. An increase in the metabolism of glucose via the pentose phosphate pathway has also been reported by McKern (3) who showed that HCG activates corpus luteum glucose-6-phosphate dehydrogenase.

The manner in which the hormone exerts these effects is largely unknown although cAMP has been variously implicated. A recent report by Vaitukaitis *et al.* (4) indicated that exogenous HCG rapidly activates soluble ovarian cAMP-dependent protein kinase, but the proenzyme activation reaches a maximum when there is as yet little change in the intracellular level of cAMP. On the other hand, increase in lactate production requires a much longer incubation period

*Abbreviations: human chorionic gonadotropin, HCG; adenosine-3',5'-monophosphate, cyclic AMP or cAMP; N^6-$O^{2'}$-dibutyryl-adenosine-3',5'-monophosphate, dbcAMP.

with HCG when glucose is used as substrate (5). These data suggest that while HCG can accelerate glycogenolysis by activating the protein kinase system, some other mechanisms might be operative in mediating the effect of HCG on subsequent steps of carbohydrate metabolism.

In this connection, the effect of HCG on the glycolytic enzyme phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC2.7.1.11) is of interest since it has been shown (6) that phosphofructokinase is the rate-limiting enzyme in the glycolytic sequence and its activation is not dependent on enzyme phosphorylation. The present investigation was undertaken to examine the possibility that HCG might stimulate glycolysis in rat ovaries by altering the catalytic efficiency of phosphofructokinase or by promoting its synthesis.

EXPERIMENTAL

Female albino rats originating from Simenson Laboratory (Gilroy, California, U.S.A.) and aged 30 days were used. These were killed by cervical fracture. The ovaries were rapidly removed and trimmed of fat in ice-cold 0.1 M Tris-HCl buffer, pH 8.0 containing 150 mM KCl, 5 mM EDTA and 10 mM 2-mercaptoethanol. They were then cut into small pieces, washed several times with cold buffer and blotted dry. The slices were then weighed and divided into the required number of groups. Each group was taken up in buffer at about 40 mg wet weight per ml and charged with the appropriate additions at the desired concentrations. These suspensions were then gassed with 95% O_2-5% CO_2 for 30 sec. Incubation was carried out at 37°. The incubation periods for the various experimental groups were as follows: HCG, 20 mins; dbcAMP, 120 mins; puromycin, 30 mins; actinomycin D, 100 mins; puromycin 30 mins, followed by HCG, 20 mins or dbcAMP, 120 mins; actinomycin D, 100 mins, followed by HCG, 20 mins. The control groups were incubated for an identical period of time as the corresponding experimental groups.

Following incubation, the ovarian slices were homogenized in a glass homogenizer and centrifuged at 40,000 xg for 10 mins in a Sorval RC2B refrigerated centrifuge. The supernatant fractions were collected, examined for protein content according to Lowry *et al.* (7) and for phosphofructokinase activity.

For measurement of phosphofructokinase activity, the coupled-assay method as described by Ling *et al.* (8) was used with no modification except that 4 mM NADH was used instead of 20 mM. The assay was run at 28°. Changes in absorbance of the reaction mixture at 340 nm was recorded as a function of time using a Hitachi Model 139 recording spectrophotometer. When the effect of HCG or dbcAMP on the auxiliary enzymes was studied, 2 mM fructose-1,6-diphosphate was used instead of fructose-6-phosphate and ATP. HCG or dbcAMP was added directly to the assay mixture to the final concentrations of 66.7 i.u./ml and 0.27 mM respectively.

Preliminary experiments indicated that the phosphofructokinase activity in the extract was unstable even when kept at ice-bath temperature. However, the rate of deterioration was linear with time. Therefore in subsequent experiments, at least three determinations were made for each extract and the

Table 1

Effect of HCG on Ovarian Phosphofructokinase Activity

Concentration of HCG (i.u./ml)	Phosphofructokinase activity (units/mg protein)	Relative percent
0	6.6 ± 0.7	100
1000	8.7 ± 0.6*	132
2000	13.9 ± 1.2*	211

* Statistically significant difference as compared with the control value ($p < 0.01$)

activity back extrapolated to zero time when the first assay was performed. One unit of enzyme activity is defined as that amount of enzyme capable of bringing about a rate of change in absorbance of 0.01 per min under the conditions used.

The results are expressed, wherever possible, as mean ± S.E.M. for 6 or more independent experiments. Statistical evaluation of the data was carried out using the paired Student t-test. Significant differences between the means (calculated as p values) are shown. No statistical significance is indicated when the p value was greater than 0.05.

RESULTS

When ovarian slices were incubated with urinary HCG and the subsequent extracts examined for their ability to oxidize NADH in the coupled-assay system, a stimulatory effect was observed. The results are shown in Table 1. The increases were statistically significant at both concentrations of HCG used ($p < 0.01$) and were tentatively attributed to an increase in the phosphofructokinase activity in the ovarian extracts. In order to demonstrate that the accelerated rate of NADH oxidation was not due to changes in the ovarian aldolase, triosephosphate isomerase and L-glycerophosphate dehydrogenase activities, fructose-1,6-diphosphate at a concentration of 2 mM was used as substrate in some experiments. Table 2 shows that HCG has no effect on these auxiliary enzymes.

Table 2

Effect of HCG on the Activity of Aldolase, Triosephosphate Isomerase and α-Glycerophosphate Dehydrogenase-catalysed Oxidation of NADH

Concentration of HCG (i.u./ml)	Reaction Rate* (Δ O.D.$_{340}$/min)
0	0.085 ± 0.001
1000	0.085 ± 0.001
2000	0.084 ± 0.001

* Data represent mean ± S.E.M. of 3 independent experiments

The nature of the hormone-induced enzyme increase was studied with the use of compounds known to inhibit protein and RNA synthesis. The data summarized in Fig. 1 show that pre-incubation with puromycin alone has no effect on the basal level of ovarian phosphofructokinase. However, such treatment decreases the sensitivity of rat ovaries to HCG administrated *in vitro*. The decrease amounts to only 18% but is statistically significant. It is of interest to note that the concentration of puromycin used has been reported (9) to be capable of completely blocking the incorporation of labelled amino acids into ovarian proteins. Preincubation with actinomycin D, on the other hand, produces no significant alteration in the activity of ovarian phosphofructokinase both with and without HCG in the incubation medium.

To examine the possibility that the action of HCG on ovarian phosphofructokinase may be mediated through cAMP, ovarian slices were incubated with dbcAMP in the presence or absence of puromycin. The results of these experiments are shown in Table 3. These data show that dbcAMP used at a cocentration of 8 mM significantly increases the level of ovarian phosphofructokinase. Puromycin

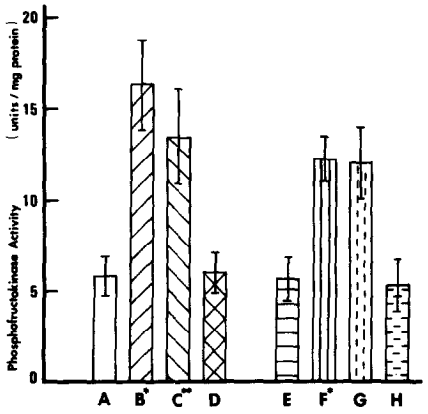

Fig. 1. Effect of Puromycin and Actinomycin D on HCG-induced Increase in Ovarian Phosphofructokinase. Ovarian slices were incubated with HCG (2,000 i.u./ml), puromycin (500 μg/ml) or actinomycin D (160 μg/ml). For experiments with puromycin: A, control; B, HCG; C, HCG + puromycin; D, puromycin. For experiments with actinomycin D: E, control; F, HCG; G, HCG + actinomycin D; H, actinomycin D. Data are presented as mean ± S.E.M. of 6 experiments. *, statistically significant difference as compared with values of control ($p < 0.001$); **, statistically significant difference as compared with values of HCG-treated ovaries ($p < 0.05$).

Table 3

Effect of dbcAMP and Puromycin on Rat Ovarian Phosphofructokinase

Concentration of dbcAMP (mM)	Concentration of puromycin (μg/ml)	Phosphofructokinase activity (units/mg protein)	Relative percent
0	0	7.64 ± 1.28	100
8	0	17.21 ± 3.24*	225
8	500	7.13 ± 2.22**	93

* Statistically significant difference as compared with the value of untreated ovary ($p < 0.01$)

** Statistically significant difference as compared with the value of dbcAMP-treated ovary ($p < 0.01$)

Table 4

Effect of HCG and cAMP on the Phosphofructokinase Activity in Ovarian Extracts

Addition	Relative percent*
None	100 ± 35
HCG	102 ± 39
cAMP	103 ± 27

* Data represent mean ± S.E.M. of 3 independent experiments

completely abolishes the stimulatory action of dbcAMP, in contrast to its effect on the HCG-induced response.

Table 4 shows the effect of HCG and cAMP when they were added directly to extracts of ovaries. It is evident that neither HCG nor cAMP can stimulate the phosphofructokinase activity in these extracts. A similar lack of effect is observable with pyromycin and actinomycin D in Fig. 1.

DISCUSSION

The results of the present study show that HCG can bring about an increase in phosphofructokinase activity in prepubertal ovaries. This is demonstrated by the fact that extracts of HCG-treated ovaries can accelerate the oxidation of NADH only when fructose-6-phosphate was used as substrate. No such changes are observed with the use of fructose-1,6-diphosphate, indicating that HCG has no effect on rat ovarian aldolase, triosephospahte isomerase and L-glycerophosphate dehydrogenase. These data suggest that an increase in phosphofructokinase activity is responsible for the HCG-stimulated lactate production in rat ovaries reported by Ahren *et al.* (9).

The stimulatory effect of HCG is only moderately affected by puromycin while actinomycin D has no effect at all, suggesting that *de novo* enzyme synthesis is not the sole factor in the activating mechanism. On the other hand, the data are consistent with the hypothesis that HCG also acts by inhibiting the degradation or inactivation of phosphofructokinase. Phosphofructokinase has been shown (6) to exist in both active and inactive aggregate forms and their interconversion is pH- and ligand-directed. The basal level of phosphofructokinase represents a balance between the biosynthetic and activation-inactivation processes. One of the activating ligands is cAMP (6). It is suggested that the cAMP formed in response to HCG administration stabilizes phosphofructokinase in its most active polymeric form in addition to promoting its synthesis. These effects are manifest in an observable increase in enzyme level. The slight inhibitory effect of puromycin is due to suppressed biosynthesis while the fact that mammalian mRNA has a relatively long life time (10) would account for the lack of effect of actinomycin D.

Since cAMP is also an allosteric effector of phosphofructokinase, the possibility that HCG and cAMP may stimulate phosphofructokinase through an allosteric mechanism must be considered. However, in the present case, this possibility appears unlikely in view of the fact that HCG and cAMP added directly to the untreated ovarian extract fail to elicit any activity changes. This contention is further supported by the finding (6) that at pH 8.0, phosphofructokinase exhibits Michaelis-Menten type of kinetics.

Ovarian slices treated with dbcAMP also show an enhancement in phosphofructokinase activity. However, this response appears to be qualitatively different from that elicited by HCG. The stimulatory action of dbcAMP is completely abolished by puromycin. A similar lack of correspondence in the action of hormone and the dibutyryl analog of its presumed 'second messenger' has previously been reported in several systems (9, 11, 12). It has been suggested (13) that the resistance of dbcAMP to phosphodiesterase is responsible for the apparent anomaly. Thus it is quite possible that in certain

tissues, the protein synthesizing machinery will be responsive to *in vitro* treatment with dbcAMP but not to hormones because of the rapid inactivation of cAMP. Our present data with HCG- and dbcAMP-induced ovarian phosphofructokinase activity are consistent with this theory, although it remains to be ascertained that dbcAMP has no stabilizing effect on phosphofructokinase. Experiments to verify this are now in progress.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the World Health Foundation (H.K.). The advice rendered by Drs. C.C. Yu and W.N. Yu in the course of this work is also gratefully acknowledged.

REFERENCES

1. Demers, L.M., Gabbe, S.G., Villee, C.A. & Greep, R.O. (1973) Biochim. Biophys. Acta 313:202-210.
2. Hamberger, S.A. & Ahren, K.E.B. (1967) Endocrinology 81:93-100.
3. McKern, K.W. (1973) Biochemistry 12:5206-5211.
4. Vaitukaitis, J.L., Lee, C.Y., Ebersole, E.R. & Lerario, A.C. (1975) Endocrinology 97:215-222.
5. Nilsson, L., Rosberg, S. & Ahren, K.E.B. (1974) Acta Endocrin. 77:559-574.
6. Mansour, T.E. (1972) in Current Topics in Cellular Regulation vol. 5, pp. 1-46, Horecker, B.L. & Stadtman, E.R., eds., Acad. Press, New York & London.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & RAndall, R.J. (1951) J. Biol. Chem. 193:265-275.
8. Ling, K.H., Paetkau, V., Marcus, F. & Lardy, H.A. (1966) in Methods in Enzymology vol. IX, pp.425-429, Wood, W.A., ed. Acad. Press, New York & London.
9. Ahren, K., Hamberger, L. & Rubinstein, L. (1968) Acta Physiol. Scand., 74:79-90.
10. Korner, A. (1970) in Control Processes in Multicellular Organisms, pp.86-107, Wolstenholme, G.E.W. & Knight, J., eds., J. & A. Churchill, London.
11. Pearlmutter, A.F., Rapino, E. & Murray, S. (1973) Endocrinology 92:679-686.
12. Burke, G., Kowalski, K. & Yu, S. (1971) Acta Endocrinol. 68:183-195.
13. Cheung, W.Y. (1970) in Role of Cyclic AMP in Cell Function, pp. 51-65, Greengard, P. & Costa, E., eds., Raven Press, New York.