STUDIES ON THE EFFECTS OF INSULIN AND ACETYLCHOLINE ON ACTIVATION OF GLYCOGEN SYNTHASE AND ON GLYCOGENESIS IN HEPATOCYTES ISOLATED FROM NORMAL FED RATS*

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SUMMARY: The addition of insulin (4.0×10^{-11} M) or acetylcholine (10^{-6} M) to isolated hepatocytes stimulated glycogen accumulation and this stimulation was more pronounced when the medium glucose was raised from 50 to 300 mg percent. Studies with [^{14}C]-glucose showed a two-fold stimulation in glycogen synthesis by the addition of insulin (4.0×10^{-11} M) or acetylcholine (10^{-6} M). A sixteen percent increase in the activity of glycogen synthase was observed in cells incubated for 10 minutes with insulin (4.0×10^{-11} M) or acetylcholine (10^{-6} M), whereas at one hour incubation a 40 percent increase in activity was observed with the same concentration of insulin or acetylcholine. The effects of insulin and acetylcholine were not additive.

It is well known that insulin is involved in glycogen synthesis in post absorptive state. The glycogen synthesis is brought about by the enzyme glycogen synthase which exists in two forms: the less active form (D) and the active form (I). Activation of this enzyme by the conversion of the "D" to the "I" form is brought about by insulin (1, 2). However, the exact mechanism of hormonal regulation of the enzyme transformation in liver is still not clear. We have previously observed that *in vitro* addition of insulin helped to maintain better cellular structure and more glycogen in isolated hepatocytes as studied by electron microscopy (3). In this communication we present evidence for net glycogen synthesis and activation of glycogen synthase in isolated hepatocytes with insulin and acetylcholine.

MATERIALS AND METHODS

Male fed Cox rats (160-200 g) were used for all these studies. Rat liver parenchymal cells were isolated by collagenase *in vitro* perfusion

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technique as described previously (4). Approximately 55-75 mg of wet weight of cells were incubated in 3 ml of Umbreit Ringer 25 mM $NaHCO_3$ buffer (4) containing 5 mM lactate and 5 mM amino acids mixture and with various concentrations of glucose (50-300 mg percent) and in the presence of insulin (4.0×10^{-11} M) and acetylcholine (10^{-6} M) at 37°C. At the end of incubation, the vial contents were placed in iced centrifuge tubes and centrifuged at 2000 rpm in an International Centrifuge for 5 minutes. The resulting pellet was used for cellular glycogen assay by the glycogen precipitation method of Good et al. (5). The precipitated glycogen was washed, hydrolyzed and assayed as glucose by the glucose oxidase method (6). Glycogen content is expressed as μmoles glucose/g. Radioactivity in glycogen was assayed after hydrolysis of glycogen to glucose which was further isolated as phenyl osazone derivative as described previously (7). Results have been computed as [^{14}C]-glucose incorporated into glycogen/g. Hepatocytes for glycogen synthase assay were similarly prepared and incubated as described above. After incubation, vial contents were centrifuged, the supernatant was decanted, the pellet was resuspended in 1 ml of ice-cold Tris (50 mM), EDTA (1 mM), 2-mercaptoethanol (25 mM) buffer and sonicated for five seconds at 50 volts with a sonifier cell disruptor (Model W185 D, Heat Systems-Ultrasonics, Inc.). The samples were centrifuged at 2000 rpm for 20 minutes in a refrigerated centrifuge and the supernatant was used to determine glycogen synthase activity by the incorporation of radioactive glucose into glycogen from UDP-[^{14}C]-glucose (8).

RESULTS AND DISCUSSIONS

Results on glycogen synthesis with various concentrations of glucose and in the presence of insulin and acetylcholine are summarized in Table I. It can be seen from this table that both insulin (4.0×10^{-11} M) and acetylcholine (10^{-6} M) stimulated glycogen synthesis in isolated hepatocytes. No net change in glycogen synthesis was observed with 50 mg percent glucose in the medium. With the increase in glucose concentration from 100-300 mg percent a signifi-

TABLE I

EFFECTS OF INSULIN AND ACETYLCHOLINE ON NET GLYCOGEN SYNTHESIS IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF GLUCOSE IN ISOLATED HEPATOCYTES FROM NORMAL FED RATS*

	CONCENTRATION OF GLUCOSE IN THE MEDIUM				
	(50 mg%)	(100 mg%)	(150 mg%)	(200 mg%)	(300 mg%)
Control	51.7 ± 6 (6)	55.2 ± 8 (6)	80.0 ± 14 (10)	88.0 ± 11 (10)	91.5 ± 12.9 (10)
Control + Insulin (20 μunits/3 ml)	52.6 ± 5 (6)	72.9 ± 10 (6)	109.9 ± 14 (10)	118.3 ± 8 (10)	128.7 ± 8.0 (10)
Control + Acetylcholine (10^{-6} M)	52.1 ± 6 (6)	74.0 ± 10 (6)	110.2 ± 10 (10)	119.4 ± 14 (10)	131.2 ± 8.5 (10)
Control + Insulin (20 μunits) + Acetylcholine (10^{-6} M)	54.1 ± 7 (6)	72.7 ± 8 (6)	110.9 ± 16 (10)	119.4 ± 14 (10)	131.1 ± 8.5 (10)

*Hepatocytes were prepared as described in Materials and Methods (4). Isolated hepatocytes had initial glycogen levels in the range of 150 ± 23 μmoles glucose/g. Approximately 55-75 mg of cells were incubated for one hour in 3 ml Umbreit Ringer bicarbonate buffer containing 5 mM lactate and 5 mM amino acids mixture and with various concentrations of glucose, insulin and acetylcholine. Values shown above (μmoles glucose/g) are mean values ± SEM of (N) observations.

cant increase in glycogen synthesis was observed in the presence of insulin and acetylcholine. The effects of insulin and acetylcholine were similar and no additive effect was observed when cells were incubated with both insulin and acetylcholine. The results on the incorporation of [^{14}C]-gluocse into glycogen are summarized in Table II. It can be seen from this table that both insulin and acetylcholine stimulated the incorporation of [^{14}C]-glucose into glycogen by two-fold. A two-fold stimulation in the incorporation of [^{14}C]-glucose into glycogen was observed with both insulin and acetylcholine when 50 mg percent glucose was used, although at this concentration, no net increase in glycogen synthesis was observed (Table I). This stimulation in the incorporation of [^{14}C]-glucose into glycogen was observed at all glucose concentrations studied, although stimulation of isotope incorporation was comparatively higher when lower concentrations of glucose were used. These results suggest that insulin is not involved in glucose transport but is involved in glycogen synthesis. We have reported in our previous studies (3) that in the absence of insulin, 800 mg percent glucose was needed to maintain initial glycogen levels. In the present studies initial glycogen levels were maintained with 20 μunits/3 ml (4.0×10^{-11} M) of insulin, and with only 200-300 mg percent glucose in the medium. Conditions under which net glycogen synthesis was observed in these studies with isolated hepatocytes in the presence of insulin are similar to normal *in vivo* post absorptive state.

Studies on the activation of glycogen synthase are summarized in Table III. It can be seen from this table that insulin (4.0×10^{-11} M) stimulated glycogen synthase activity in 10 minutes by sixteen percent. Continued incubation for one hour with insulin (4.0×10^{-11} M) showed a further increase in activity of glycogen synthase by forty percent. This is the first report clearly showing the effect of insulin on net glycogen synthesis and activation of glycogen synthase in isolated hepatocytes at the physiological concentration of insulin (4.0×10^{-11} M). Although similar results are observed with acetylcholine, concentration required is quite high (10^{-6} M). Miller and

TABLE II

EFFECTS OF INSULIN AND ACETYLCHOLINE ON GLYCOGEN SYNTHESIS IN ISOLATED HEPATOCYTES FROM NORMAL FED RATS* AS MEASURED BY ^{14}C-GLUCOSE INCORPORATION INTO GLYCOGEN

	CONCENTRATION OF GLUCOSE IN THE MEDIUM			
	(50 mg%)	(100 mg%)	(200 mg%)	(300 mg%)
Control	9,627 ± 1,752 (6)	12,308 ± 2,249 (6)	30,742 ± 3,510 (4)	40,982 ± 4,930 (10)
Control + Insulin (20 μunits/3 ml)	17,801 ± 1,591 (6)	24,840 ± 2,431 (6)	46,404 ± 3,690 (4)	60,658 ± 5,580 (10)
Control + Acetylcholine (10^{-6} M)	16,773 ± 1,652 (6)	26,268 ± 2,319 (6)	55,156 ± 4,580 (4)	60,348 ± 4,590 (10)
Control + Insulin (20 μunits/3 ml) + Acetylcholine (10^{-6} M)	17,262 ± 1,670 (6)	27,921 ± 2,416 (6)	55,935 ± 6,420 (4)	60,489 ± 5,080 (10)

*Hepatocytes were prepared as described in Materials and Methods (4). Isolated hepatocytes had initial glycogen levels in the range of 150 ± 23 μmoles glucose/g/hr. Approximately 55-75 mg of cells were incubated for one hour in 3 ml of Umbreit Ringer bicarbonate buffer containing 5 mM lactate and 5 mM amino acids mixture and with various concentrations of glucose containing approximately 1 μc of ^{14}C-glucose (per 100 mg) and with insulin and acetylcholine. Values shown above are (CPM/g) mean values ± SEM of (N) observations.

TABLE III

EFFECTS OF INSULIN AND ACETYLCHOLINE ON GLYCOGEN SYNTHASE ACTIVITY IN THE PRESENCE OF 300 MG PERCENT OF GLUCOSE IN ISOLATED HEPATOCYTES FROM NORMAL FED RATS*

	INCUBATION TIME (10 min)	INCUBATION TIME (60 min)
Control	5.9 ± 0.6 (4)	6.8 ± 0.3 (4)
Control + Insulin (20 μunits/3 ml)	6.9 ± 0.8 (4)	9.2 ± 0.9 (4)
Control + Acetylcholine (10^{-6} M)	6.8 ± 0.7 (4)	9.8 ± 1.1 (4)
Control + Insulin (20 μunits/3 ml) + Acetylcholine (10^{-6} M)	7.1 ± 0.85 (4)	9.0 ± 0.75 (4)

*Hepatocytes were prepared as described in Materials and Methods (4). Isolated hepatocytes had initial glycogen levels in the range of 150 ± 23 μmoles glucose/g. Approximately 55-75 mg of cells were incubated for one hour in 3 ml of Umbreit Ringer bicarbonate buffer containing 5 mM lactate and 5 mM amino acids mixture and 300 mg% glucose and with insulin and acetylcholine. Values were expressed as mean ± SEM of (N) observations percent glycogen synthetase I increase over glycogen synthetase D.

Larner (1) have shown stimulation of glycogen synthase activation by insulin in the isolated perfused liver and have observed that this effect was maximal for 6-15 minutes only. However, in the present studies with isolated hepatocytes, we were able to observe maximal effects of insulin for one hour and this stimulation of glycogen synthase activity is far greater than that observed in perfused liver (1). This may be due to other cell types which may destroy insulin being absent in our hepatocytes preparation. The data presented here clearly shows that glycogen synthesis and activation of glycogen synthase and not glucose transport is mediated by insulin and perhaps by acetylcholine.

The control of glycogen synthesis by insulin (1-3, 9) has remained controversial due to the lack of direct in vitro evidence and due to several in vivo (10) and in vitro (11) negative reports. However the results presented here and those previously reported from this laboratory (3) and also by Larner et al. (1) demonstrate that insulin functions directly in glycogen synthesis through the activation of glycogen synthase and several mechanisms for this activation have been proposed (1).

Shimazu et al. (12, 13) have shown that stimulation of parasympathetic nerves caused a marked increase in the activity of glycogen synthase in the liver followed by a decrease in blood glucose. These observations are in agreement with the present studies. It is known that in vivo parasympathetic stimulation causes the release of both insulin (14, 15), and acetylcholine (12, 13) which may in turn stimulate glycogen synthase as observed in the present in vitro studies. Since we have, in this study, been able to show that insulin as well as acetylcholine stimulates glycogen synthase in isolated hepatocytes, it may be of interest to consider the employment of cholinergic drugs - parasympathomimetics, to investigate the exact mechanism of the hormonal regulation of hepatic glycogen synthase which might be implicated in the receptor proteins. It would be worthwhile to investigate if this activation is brought about by mechanisms which may include a role for cyclic AMP or cyclic GMP.

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REFERENCES

1. Miller, T.B. and Larner, J.: J. Biol. Chem. 248:2483, 1973.
2. Blatt, L.M. and Kithan, Kim: J. Biol. Chem. 246:7256, 1971.
3. Wagle, S.R., Ingebretsen, W.R. and Sampson, L.: Biochem. Biophys. Res. Comm. 53:937, 1973.
4. Ingebretsen, W.R. and Wagle, S.R.: Biochem. Biophys. Res. Comm. 47:403, 1972.
5. Good, R.A., Kramer, H. and Samogyi: J. Biol. Chem. 100:485, 1933.
6. Hagget, A. and Nixon, S.Z.: Lancer 2:268, 1957.
7. Wagle, S.R. and Ashmore, J: J. Biol. Chem. 236:2828, 1961.

8. Thomas, J.A., Schlender, K.K. and Larner, J.: Annals of Biochem. 25: 486, 1968.
9. Kreutner, W. and Goldberg, N.B.: Proc. Nat. Acad. Sci. (US) 58:1515, 1967.
10. DeWulf, H. and Hers, H.G.: Eur. J. Biochem. 6:558, 1968.
11. Glinsman, W., Pauk, G. and Hern, E.: Biochem. Biophys. Res. Comm. 39:774, 1970.
12. Shimazu, T., Fukuda, A. and Barr, T.: Nature 210:1178, 1966.
13. Shimazu, T.: Science 156:1256, 1967.
14. Frohman, L.A., Edzini, E.Z. and Javid, R.: Diabetes 16:443, 1967.
15. Kanetas, A., Kosaka, K. and Nakas, K.: Endocrinology 80:530, 1967.