Vol. 96, No. 2, 1980
September 30, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 748-755

REGULATION OF GLUCOSE-6-P DEHYDROGENASE ACTIVITY IN PRIMARY RAT HEPATOCYTE CULTURES

Larry Winberry, Roderick Nakayama, Ronald Wolfe and Darold Holten

Department of Biochemistry
University of California
Riverside, California 92521

Received August 18, 1980

Summary:

The glucose-6-P dehydrogenase specific activity in rat hepatocytes increases approximately 10-fold when the cells are placed into culture for three days. The induction requires insulin with maximum enzyme levels occurring at 10^{-7} M. Pulse-labeling experiments revealed a 10-fold increase in the enzyme's relative rate of synthesis after only 8 hours in culture.

Introduction

An important aspect of fatty acid metabolism in mammalian liver is the induction of a number of key lipogenic enzymes by nutritional and/or hormonal factors (1). Two of these enzymes, glucose-6-P dehydrogenase (G6PD, E.C. 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44) are induced 25- and 5-fold respectively when rats are fasted and then refed a high-carbohydrate, fat-free diet (2-4). The induction of both enzymes has been shown to result from an increase in the rate of enzyme synthesis (5-8). To date no definitive results have been obtained as to the exact nature of the nutritional or hormonal signals which regulate the synthesis of these two lipogenic enzymes. In this report, we described the use of cultured primary rat hepatocytes to investigate the hormonal requirements for the induction of G6PD.

Materials

Collagenase (Type I) was obtained from Worthington. Bovine serum albumin, glucagon, hydrocortisone, HEPES,[1] insulin, T3,[1] theophylline, trans-

Abbreviations

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; T3, 3,3',5-triiodo-L-thyronine; Bt_2cAMP, dibutyryl adenosine 3'5'-cyclic monophosphate; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

0006-291X/80/180748-08$01.00/0

ferrin, secretin, and somatostatin were obtained from Sigma. Fetal calf serum, penicillin, and streptomycin were obtained from Grand Island Biological Co. Bt_2cAMP[1] was obtained from Boehringer-Mannheim, Gentamycin from Schering, and newborn calf serum from Biocell. All other materials were as described previously (8).

Methods

Assays for G6PD and protein, treatment of animals, and the composition of their diets were as previously described (5). One unit of G6PD is defined as that amount of enzyme capable of producing 1 μmol of NADPH/min at 30°C and pH 8.0. Specific activity is expressed as units of enzyme/mg protein.

Preparation of Hepatocytes and Culture Conditions

Hepatocytes were isolated from young male Sprague-Dawley rats by perfusion of the liver with collagenase as described by Berry and Friend (9) except that calcium-free Krebs original Ringer phosphate buffer was used for the perfusions. The isolated cells were washed several times with perfusion buffer by low speed centrifugation and then with a modified Hams F-12 medium, referred to as Hams F-12/SA, which was supplemented with 0.4 mM ornithine (no arginine), 5 mM glucose, 5 mM pyruvate, 0.28 mM HEPES, 5 mg/ml transferrin, 10 mg/liter phenol red, amino acid concentrations similar to the LHSA medium described by Oliver et al. (10), and 0.05 mg/ml gentamycin.

After sedimenting, the cells were diluted to 2% (v/v) with Hams F-12/SA medium supplemented with 10% fetal calf (FCS) or newborn calf serum (NCS), 10^{-7} M insulin, 10^{-6} M hydrocortisone, and 10^{-6} M thyroxine. The cells were plated into either 60 mm or 100 mm plastic tissue culture dishes (Falcon Plastics) by using 3 ml or 5 ml of cell suspension, respectively. The cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO_2. After 4 hours, the medium containing dead and unattached cells was removed by aspiration and replaced with serum-free Hams F-12/SA medium supplemented with 0.3% BSA[1] (fatty acid-free), 5 mM acetate, and the various hormones to be tested. Control cultures were hormone-free. Fresh medium was provided at 24 hour intervals. At the first 24 hour feeding, the gentamycin was replaced with penicillin and streptomycin (100 μg/ml each).

Prior to harvesting, culture dishes were washed extensively with cold 0.15 M KCl. Using a rubber policeman, the cells were scraped into 0.5 ml cold 0.15 M KCl and stored at -70°C. For enzyme assays, the thawed cell suspensions were sonicated and centrifuged at 14,000 x g for 60 min at 4°C.

Determination of Glucose-6-P Dehydrogenase Relative Rate of Synthesis

Hepatocytes were isolated and placed into culture as described above. The medium was removed and replaced with fresh medium containing 20 μCi/ml ^{3}H-leucine. After 60 min the cells were harvested and the G6PD was isolated by immunoprecipitation as previously described (8).

RESULTS AND DISCUSSION

Our laboratory would like to determine to what extent hormones are involved in the regulation of G6PD and 6PGD levels in mammalian liver. The ability to pursue this goal requires the development of a more simplified

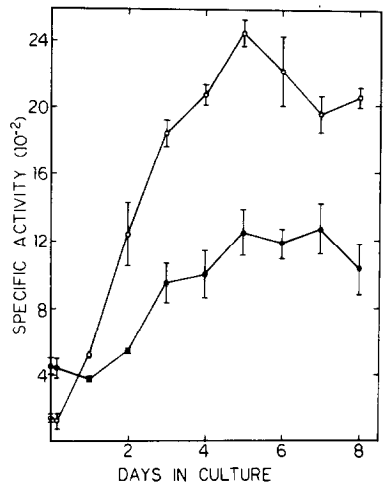

Figure 1. Induction of G6PD and 6PGD Activity in Cultured Hepatocytes.

Hepatocytes were isolated form the liver of a fasted rat and placed into culture in Hams F-12/SA medium which was supplemented with 10% FCS, 10^{-7} M insulin, 10^{-6} M hydrocortisone, and 10^{-6} M thyroxine. At the indicated times the cells were harvested and the enzyme activities determined as in the Methods section. The results shown are the mean $\pm$ S.D. for three replicate cultures. (Open circles: G6PD activity; closed circles: 6PGD activity).

system than the animal model which we have used in previous studies. The data presented in Figure 1 demonstrates the feasibility of using adult hepatocytes in primary culture to study this induction process. Under the culture conditions employed in this experiment, the specific activities of G6PD and 6PGD had increased approximately 10- and 3-fold respectively.

In order to optimize the magnitude of this induction, the hepatocytes had been isolated from a fasted rat and cultured in the presence of insulin, hydrocortisone, and T3, all of which have been implicated in the regulation of lipogenic enzyme levels (1). Our next objective was to determine which, if any, of these hormones were actually responsible for this induction. Following a 4 hour plating period, the cells were incubated for 3 days in the presence or absence of all three hormones (Fig. 2). Clearly, the induction of G6PD depends upon the continued presence of one or more of the hormones. It should be noted that these hepatocytes had been plated in the presence of the hormones to ensure maximal cell attachment. The 4 hour exposure to these conditions appears to be sufficient to cause a significant induction

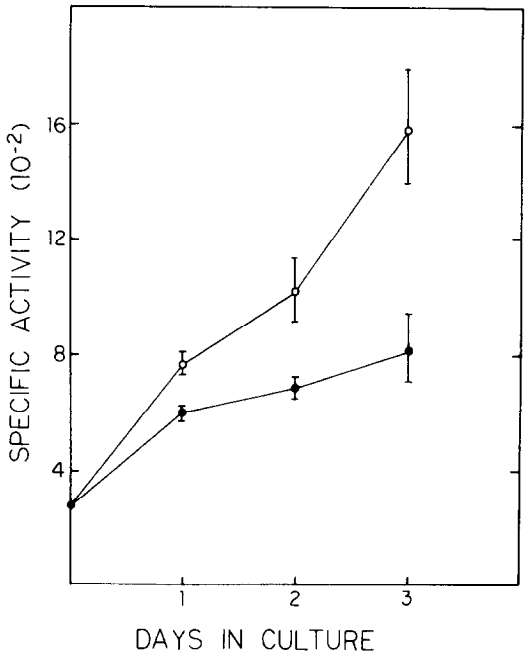

Figure 2. G6PD Induction in Defined Media.

Hepatocytes were prepared from a fasted rat and plated as in the legend to Figure 1. Four hours after plating, the media was replaced with serum-free Hams F-12/SA which was supplemented with 0.3% fatty-acid free BSA and either contained (open circles) or lacked (closed circles) insulin, hydrocortisone, and thyroxine at the concentrations present in the plating media. The results shown represent the mean ± S.D. for five replicate cultures.

even in the control-"hormone free" cultures and this causes a significant reduction in the apparent hormonal-dependency of this process. In spite of this limitation, we felt this approach would allow the determination of the hormones required for the induction of G6PD.

Shown in Figure 3 are the results obtained when hepatocytes were cultured in the presence of various hormonal stimuli for 3 days. It is obvious from these data that insulin is primarily responsible for the induction of G6PD in these cells. From dose response curves, we have determined that the stimulation by insulin is maximal at 10^{-7} M (data not shown). Several other lipogenic enzymes have been shown, using cultured hepatocytes, to be induced by insulin. These include fatty acid synthetase (11), malic enzyme (12), and ATP-citrate lyase (13). These observations are consistent with the

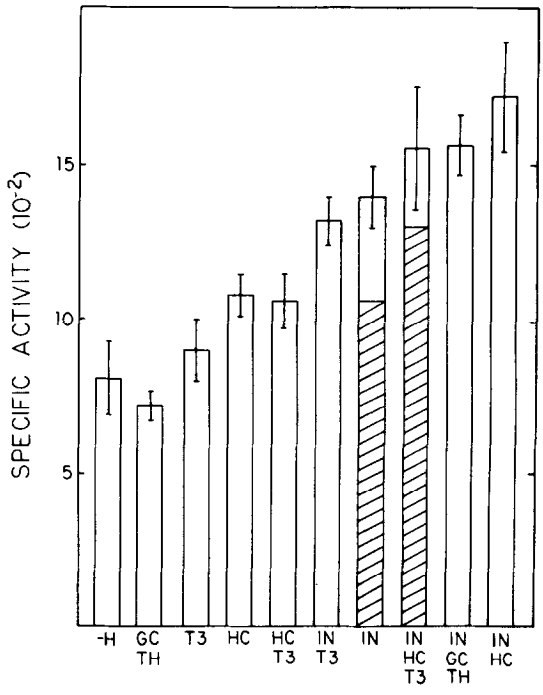

Figure 3. G6PD Induction in Defined Media: Hormonal Requirements.

Hepatocytes were plated in hormone-supplemented media for 4 hours as described in the legend for Figure 2. Thereafter the cells were cultured in defined media containing various hormones or hormone combinations with daily media changes. Three days after plating the cells were harvested and the G6PD specific activities determined. The symbols refer to the following culture conditions: -H, no hormones; GC, 10^{-8} M glucagon; HC, 10^{-6} M hydrocortisone; IN, 10^{-7} M insulin; T3, 10^{-6} M T3; and TH, 4 x 10^{-4} M theophylline. The results shown are the mean $\pm$ S.D. for five replicate cultures. The hatched bars represent results obtained when serum was present in the culture media for the entire 3 days.

lack of induction of lipogenic enzymes in starved-refed rats made diabetic by injection of alloxan or streptozotocin (14).

The data in Figure 3 also indicate a slight (20-30%) but reproducible stimulation of G6PD activity by hydrocortisone which occurs both in the presence or absence of insulin. Similar results have been reported for ATP-citrate lyase (13) and Bouilon and Berdanier (15) have recently shown that glucocorticoids increase the induction of G6PD in adrenalectomized rats which have been fasted and refed a high carbohydrate diet. Thus glucocorticoids may play a role in regulating the levels of G6PD in liver.

Studies with thyroidectomized rats (16,21) and cultured chick liver cells (12) have suggested that thyroxine may be involved in the induction of lipogenic enzymes. However, as can be seen in Figure 3, T3 was found to be ineffective as an inducer of G6PD. This result is not surprising when one considers the recent results of Spence et al (17,13) who demonstrated that hepatocytes must be prepared from thyroidectomized animals in order to observe a T3-dependent increase in glucokinase or ATP-citrate lyase activities. Obviously we must repeat the T3 experiment under those conditions before reaching a conclusion regarding its involvement in the induction of G6PD.

Our previous studies have shown that glucagon or cAMP was capable of decreasing the G6PD level in carbohydrate refed rats (18) due to a repression in the rate of G6PD synthesis (19). The synthesis of malic enzyme (12) and fatty acid synthetase (20) have also been shown to be regulated by cAMP. Thus, it was somewhat surprising that glucagon was not able to decrease the induction of G6PD in cultured hepatocytes. In other studies (data not shown) Bt_2cAMP caused a slight inhibition of the induction of G6PD in cultured hepatocytes but the effect was much smaller than we had observed in intact rats (18). The glucagon insensitivity may be due to the fact that in culture, part of the capacity (signal?) for G6PD induction is missing. For example, in vivo, G6PD can be induced to specific activities of approximately 0.5-0.6 units/mg protein whereas in cultured hepatocytes the activity attained following induction is only 0.2-0.3 units/mg protein (Fig. 1). It appears that the culture environment only partially mimics the in vivo state and this may indicate the participation of several independent mechanisms in the regulation of this enzyme, one of which appears to be nonfunctional or absent in the cultured cells. Consistent with this proposal is the observation (data not shown) that hepatocytes prepared from a fasted-refed rat exhibit, at the time of plating, the G6PD specific activity expected for the in vivo induced state (0.5). However, by day 6 in culture, this activity had declined to and stabilized at a level equal to the induced level observed in Figure 1 (0.2).

TABLE 1
SYNTHESIS OF G6PD IN CULTURED HEPATOCYTES

TIME (hrs.)	SPECIFIC ACTIVITY	DPM in G6PD	DPM in TOTAL	RELATIVE RATE of SYNTHESIS
2.5	0.010	1018	2.51×10^7	0.004%
4.5	0.008	1096	9.86×10^6	0.010%
8.5	0.004	796	1.59×10^6	0.050%

Hepatocytes were isolated from the liver of a fasted rat and placed into culture as described in the legend for Figure 1. At the indicated times, the cells were labeled for 90 minutes with ^{3}H-leucine (20 uc/ml). The labeled cells were harvested, sonicated and a postmicrosomal fraction prepared. The radiolabeled G6PD was isolated by immunoprecipitation and the dissociated precipitates subjected to analysis on SDS-gels. The radioactivity in the gel which corresponded to G6PD was determined and is reported above. The radioactivity incorporated into total TCA-insoluble material is reported in the column labeled DPM in TOTAL.

A second possibility is that the glucagon or Bt_2cAMP do not act directly on liver. For example, glucagon increases lipolysis from adipose tissue and the free fatty acids released may cause the decrease in G6PD synthesis in liver. The cultured hepatocytes can be used to explore this and other possibilities.

In order to determine whether the increased levels of G6PD in cultured cells was due to an increase in its rate of synthesis as has been found in live animals during induction (8), we labeled cells with [^{3}H]leucine and isolated the newly synthesized G6PD by immunoprecipitation. As can be seen in Table 1, there was an approximately 10-fold increase in the relative rate of synthesis of G6PD within 8.5 hours of plating. This increase precedes, as expected, any increase in enzyme specific acitvity and can fully account for the induction of G6PD.

These studies demonstrate the usefulness of this culture system for providing additional insight into the mechanisms which regulate this enzyme, and perhaps other lipogenic enzymes, during their nutritional and/or hormonal induction. Our current data however, suggest that insulin is the most important hormone regulating the synthesis of G6PD in rat liver.

Acknowledgment

This research was supported in part by Public Health Service grant no. Am-13324. L. W. was supported by Public Health Service Training Grant AM-07310.

References

1. Volpe, J. J., and Vagelos, P. R. (1976) Physiol. Rev. 56, 338-417.
2. Tepperman, H. M., and Tepperman, J. (1963) Adv. Enzyme Regul. 1, 121-136.
3. Glock, G. E., and McLean, P. (1955) Biochem. J. 61, 390-396.
4. Huggins, C., and Yao, F. (1959) J. Exp. Med. 110, 899-919.
5. Rudack, D., Chisholm, E. M., and D. Holten (1971) J. Biol. Chem. 246, 1249-1254.
6. Rudack, D., Gozukara, E. M., Chisholm, E. M., and Holten, D. (1971) Biochim. Biophys. Acta 252, 305-313.
7. Procsal, D., Winberry, L., and Holten, D. (1976) J. Biol. Chem. 251, 3539-3544.
8. Winberry, L., and Holten, D. (1977) J. Biol. Chem. 252, 7796-7801.
9. Berry, N. M., and Friend, D. S. (1969) J. Cell Biol. 43, 506-520.
10. Oliver, I. T., Edwards, A. M., and Pitot, H. C. (1978) Eur. J. Biochem. 87, 221-227.
11. Alberts, A. W., Ferguson, K., Hennessy, S., and Vagelos, P. R. (1974) J. Biol. Chem. 249, 5241-5249.
12. Goodridge, A. G. and Adelman, T. G. (1976) 251, 3027-3032.
13. Spence, J. T., Pitot, H. C., and Zalitis, G. (1979) J. Biol. Chem. 254, 12169-12173.
14. Lakshmanan, M. R., Nepokroeff, C. M., and Porter, J. W. (1972) Proc. Natl. Acad. Sci. 69, 3516-3519.
15. Bouillon, D. J., and Berdanier, C. D. (1980) J. Nutr. 110, 286-297.
16. Roncari, D. A. K. and Veereraghaven, K. (1975) J. Biol. Chem. 250, 4134-4138.
17. Spence, J. T. and Pitot, H. C. (1979) J. Biol. Chem. 254, 12331-12336.
18. Rudack, D., Davie, B. and Holten, D. (1971) J. Biol. Chem. 246, 7823-7824.
19. Rudack-Garcia, D. and Holten, D. (1975) J. Biol. Chem. 250, 3960-3965.
20. Volpe, J. J. and Marasa, J. C. (1975) Biochim. Biophys. Acta 380, 454-472.
21. Mariash, C. N., Kaiser, F. E. and Oppenheimer, J. H. (1980) Endocrinology 106, 22-27.