Vol. 149, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1987 Pages 118-124

DEPENDENCE OF ATP CITRATE LYASE ACTIVITY ON CELL DENSITY OF ISOLATED RAT HEPATOCYTES

Anne Voss, Greg Reinhart, Susan Hogan and Karla Roehrig

Department of Food Science and Nutrition
The Ohio State University; Columbus, Ohio 43210

Received September 2, 1987

SUMMARY: The activities of two enzymes involved in the lipogenic process, ATP citrate lyase and NADP-linked malic enzyme were evaluated as a function of cell density in isolated rat hepatocytes. The activity of ATP citrate lyase was profoundly affected by cell density with the activity/cell being higher at low cell densities than at high cell densities. Malic enzyme was not similarly affected, nor was cellular ATP content. The effect was observed regardless of dietary state but was most dramatic with hepatocytes from fasted-refed rats. Both an activator and an inhibitor of ATP citrate lyase have been isolated from conditioned medium from cells at low density and at high density, respectively. The activator fraction was heat stable while the inhibitor fraction was heat labile, and both factors had molecular weights in excess of 10,000 daltons.

Hepatocytes in culture have been reported to show density-dependent growth (1), proliferating more slowly as the number of cells/ml increases. For cultured hepatocytes, evidence has been presented that cell density alters cell growth and certain cellular functions (2). A membrane protein has been isolated by Nakamura et al. (3) which may be involved in the cell density effect. In order to proliferate, cells must be able to synthesize new cell membranes and therefore would be expected to have enhanced rates of lipid synthesis. Beynen and Geelen reported elevated rates of lipogenesis with decreasing cell density (4) in freshly isolated hepatocytes. A similar finding was also reported by Jurin and McCune (5) who provided evidence for the existence of an inhibitor of fat synthesis produced by isolated hepatocytes at high density.

0006-291X/87 $1.50

The purpose of the work reported here was to determine the effects of cell density on two lipogenic enzymes, ATP citrate lyase and NADP-linked malic enzyme, the persistance of these effects in different dietary states and the mechanism(s) whereby alteration in enzyme activity might occur.

MATERIALS AND METHODS

Animals: Male, Sprague-Dawley rats were housed in wire-bottomed galvanized cages in a controlled environment animal room with a 7 AM to 7 PM dark cycle in accordance with NIH Guidelines. The diet (6) and the fasting and fasting-refeeding cycle (7) have been previously described.

Hepatocyte isolation: Hepatocytes were isolated from diethyl ether anaesthetized rats according to the procedures of Seglen (8) as modified by Geelen (9). After washing, cells were counted in an American Optical hemocytometer. Cell viability was determined by trypan blue dye exclusion and periodically confirmed by measuring cellular ATP levels in perchloric acid extracts of the cells (10). The viability was 91.5%, 91.4% and 92.5% for cells from fed, fasted and fasted-refed rats, respectively.

Factor isolation: Conditioned medium was prepared by incubating appropriately diluted hepatocytes from fed rats in Krebs Ringer bicarbonate buffer pH 7.4 containing 1% bovine serum albumin and 11mM glucose for 1 hour at 37°C in an atmosphere of 95% O_2/5% CO_2. At the end of the incubation, cells were pelleted by centrifugation (2,500 x g, 10 minutes), and the cell free medium was stored in aliquots at -20°C. Boiled medium was obtained by placing aliquots of the cell-free conditioned medium in a boiling water bath for 4 minutes then centrifuging (2,500 x g, 5 minutes) to remove precipitated protein. Conditioned medium from cells at high density (10E7) or low density (10E5) was subjected to filtration on Amicon Ultrafilters using YM10 hydrophilic membranes. After filtration, the retentate was reconstituted to its original volume while the filtrate was used as it was.

Enzyme Assays: ATP citrate lyase activity was assayed spectrophotometrically as previously described (11). The method of Ochoa (12) was used to assay malic enzyme except that 0.25 M Tris-Cl pH 7.4 was substituted for glycylglycine and the reaction volume was reduced to 1 ml.

Materials: All biochemical reagents were purchased from Sigma Chemical Co., St. Louis.

RESULTS AND DISCUSSION

During the course of evaluating ATP citrate lyase activity in hepatocytes, an apparent inverse correlation between cell density and enzyme activity was noted. Accordingly, we began a systematic study of the effect of cell density on ATP citrate lyase in hepatocytes from fed, fasted and fasted-refed rats. A

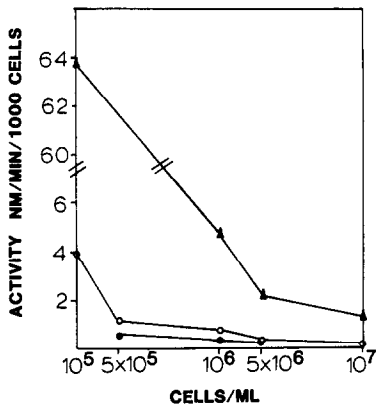

Figure 1. Cell density effects on ATP citrate lyase activity/1000 cells. Isolated hepatocytes are from livers of fed (○), fasted (●) or fasted-refed (▲) rats. Values are averages of duplicate determinations for a three replicates. Cells were diluted to the appropriate concentrations, sonicated then assayed for enzyme activity and ATP.

preliminary report has been presented (13). Activity of the enzyme/cell was highest in cells at low density (10E5 cells/ml) and lowest at high cell density (10E7 cells/ml) (Figure 1). At any given cell density, the well-known dietary dependence of ATP citrate lyase (14) was observed.

To determine whether these observations might be a protein dilution artifact, rat liver was homogenized in 5 volumes of .3M mannitol and diluted to approximate the levels of cells in the density experiments (assuming 10E8 cells/gm in intact liver). The activity corrected for dilution remained constant and was approximately equal to values for enzyme activity in the cells maintained at 10E7 cells/ml. Further, the total ATP concentration in hepatocytes was 4.67 ± 1.23 mM (average group means ± S.E.M for 17 preparations) regardless of the dietary state or cell density. Jurin and McCune reported (5) an ATP level of 2.2 umoles/gm wet weight regardless of cell density.

The activity of NADP-linked malic enzyme was also determined in hepatocytes from fed rats as a function of cell density (Table 1). Its activity did not change over the density range measured.

Table 1. Activity of NADP-linked malic enzyme in isolated hepatocyte preparations of different cell densities

Cell density	Activity (nanomoles/min/10^3 cells)
$1x10^5$	$4.8x10^{-2}$
5x105	$3.5x10^{-2}$
1x106	$5.2x10^{-2}$
5x106	$5.1x10^{-2}$
1x107	$4.8x10^{-2}$

Hepatocytes were prepared from livers of fed rats. Values are the averages of duplicate determinations from two experiments.

The lack of a density effect on malic enzyme activity, ATP levels or ATP citrate lyase activity in diluted homogenates suggested that the phenomenon was not an artifact and that only specific enzymes were affected.

It has been suggested (5) that the cell density effect on lipogenesis might be mediated by an inhibitor secreted into the media by the cells themselves. To test this hypothesis, cell-free conditioned medium (CFCM) from hepatocytes incubated 1 hour at high density (10E7 cells/ml) or low density (10E5 cells/ml) was added to freshly isolated hepatocytes and incubated at 10E6 cells/ml for various periods of time. The data in Figure 2A show

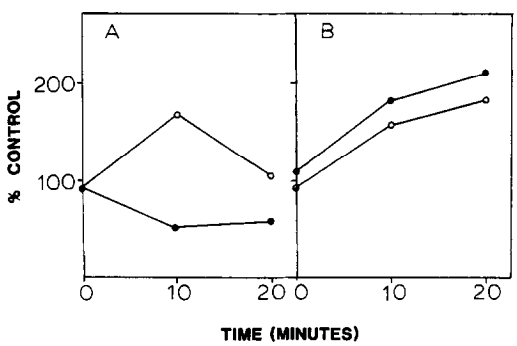

Figure 2. Effect of conditioned medium from cells at high or low density on activity of ATP citrate lyase in hepatocytes from fed rats. Values are the average of duplicate determinations for two experiments. Panel A. Hepatocytes (.3ml, 10E6 cells/ml) were incubated with 1 ml of CFCM from cells at high density (●) or low density (○) plus 1.7ml of fresh media for 0, 10 or 20 minutes at 37°C in a Dubnoff shaker. Values are compared to control incubations where CFCM was replaced with 1ml of virgin medium. Panel B. Hepatocytes were incubated as for Panel A except that CFCM and control medium were boiled as described in methods.

that CFCM from cells at high density apparently contained an inhibitor of ATP citrate lyase activity while cells at low density contained an activator. The heat stability of the two factors was determined by incubation of boiled CFCM with the hepatocytes (Figure 2B). The activator was heat stable, while the inhibitor was heat labile. Further, CFCM from cells at high density appeared to have contained both the inhibitor and the activator since boiled high density CFCM produced activation of ATP citrate lyase. Both the activator and the inhibitor in unfractionated CFCM were stable to long term (nearly 5 months) storage at -20°C.

In the work by Jurin and McCune (5), CFCM from cells at high density had an inhibitory effect on fatty acid and cholesterol synthesis, but CFCM from cells at low density had no effect. The difference may be that we have added CFCM to hepatocytes at an intermediate density (10E6 cells/ml). Since CFCM from hepatocytes at high density apparently contained both an activator and an inhibitor, it would seem that the effects of the inhibitor on ATP citrate lyase and lipogenesis may be more potent than the activator.

It could be argued that inhibition of ATP citrate lyase by CFCM from cells at high density could be a function of the build-up of unidentified inhibitory metabolic end-products. Activation by CFCM from cells at low density cannot be accounted for in this manner, however, because the comparisons were made to oxygenated medium which contained glucose and bovine serum albumin that had never been incubated with cells. The two types of CFCM were fractionated on an Amicon Ultrafilter. Both factors had molecular weights in excess of 10,000 daltons (Figure 3A and 3B) making it unlikely that the effects are due to metabolic end-products.

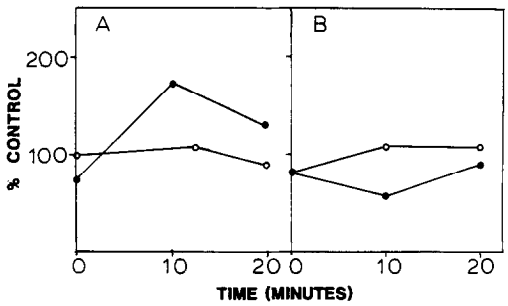

Figure 3. Effect of molecular weight fractionation of CFCM from cells at high or low density on ATP citrate lyase activity in hepatocytes from fed rats. Values are duplicate determinations for two experiments. Panel A. CFCM from cells at low density was fractionated into >10K daltons (●) or <10K daltons (○) and incubated with cells as described in Figure 2. Panel B. The same as Panel A above except that CFCM was from cells at high density.

It is increasingly evident that many types of cells release factors which can act in an autocrine manner to alter their own metabolism (15). Indeed, we have recently shown that ATP citrate lyase in rat hepatocytes is rapidly activated by transforming growth factor β (16). Others have shown that epidermal growth factor alters the phosphorylation state of the enzyme (17). The term chalone has been suggested for inhibitory factors (18). The existence of such factors which can inhibit the proliferation of cultured hepatocytes has been reported (19,20). Since liver has the capacity to regenerate, it is likely that this is a dynamic process requiring both positive and negative regulatory factors. However, prior to hepatocyte proliferation, lipogenesis would be required to provide lipid for new membrane synthesis. Therefore, it is not surprising that lipogenesis and some of the enzymes in the lipogenic pathway might be regulated by these factors. The nature of the regulation of the activity of ATP citrate lyase by these factors is yet to be determined.

ACKNOWLEDGEMENTS

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University. Journal article 158-87. This work was also supported in part by USPHS GRANT GM29255.

REFERENCES

1. Nakamura, T., Tomita, Y. and Ichihara, A. (1983) J. Biochem. 94, 1029-1035.
2. Nakamura, T., Yoshimoto, K., Tomita, Y. and Ichihara, A. (1983) Proc. Natl. Acad. Sci. USA. 80, 7229-7233.
3. Nakamura, T., Nakayama, Y. and Ichihara, A. (1984) J. Biol. Chem. 259, 8056-8058.
4. Beynen, A. and Geelen, M. (1984) Internatl. J. Biochem. 16, 105-107.
5. Jurin, R. and McCune, S. (1985) J. Cell. Physiol. 254, 442-448.
6. Allred, J. and Roehrig, K. (1973) J. Biol. Chem. 248, 4131-4133.
7. Uhal, B. and Roehrig, K. (1982) Biosci. Rep. 2, 1003-1007.
8. Seglen, P. (1976) Meth. Cell Biol. 13, 29-83.
9. Geelen, M., Beynen, A., Christiansen, R., Lepreau-Jose, M. and Gibson, D. (1978) FEBS Let. 95, 326-330.
10. Hohorst, H., Kreutz, F. and Bucher, T. (1959) Biochem. Zeit. 332, 18-46.
11. Roehrig, K., Nestor, K. and Palmquist, D. (1987) Comp. Biochem. Physiol. (in press).
12. Ochoa, S. (1955) Meth. Enzymol. 1, 739-753.
13. Voss, A. and Roehrig, K. (1987) Fed. Proc. 46, 2007.
14. Gibson, D., Lyons, R., Scott, D. and Muto, Y. (1972) Adv. Enzyme Regul. 10, 187-204.
15. Sporn, M. and Roberts, A. (1985) Nature 313, 745-747.
16. Reinhart, G. and Roehrig, K. (1987) Mol. Cell. Biochem. (in press).
17. Holland, R. and Hardie, D. (1985) FEBS Let. 181, 308-312.
18. Potter, V. (1983) Prog. Nuc. Acid. Res. Mol. Biol. 29, 161-173.
19. DePaermentier, F., Barbason, H. and Bassleer, R. (1979) Biol. Cellulaire 34, 205-212.
20. McMahon, J., Farrelly, J. and Iype, P. (1982) Proc. Natl. Acad. Sci. USA 79, 456-460.