Effect of prior nutritional status on the activity of lipogenic enzymes in primary monolayer cultures of rat hepatocytes

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Effect of prior nutritional status of the animal on the activity of lipogenic enzymes and the fatty acid content of cultured hepatocytes was investigated. Hepatocytes were isolated from rats that were starved for 24 h ('starved') or continuously fed ('fed'), or starved for 48 h and then re-fed for 48 h ('re-fed') with a carbohydrate-rich fat-free diet, and maintained as monolayer cultures for 96 h in a serum-free glucose-rich medium (Waymouth's MB752/1) supplemented with insulin, dexamethasone and tri-iodothyronine. The fatty acid content and the activities of acetyl-CoA carboxylase, fatty acid synthase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were determined initially at 3 h after plating and then every 24 h. Initially the activities of all the four enzymes were highest in hepatocytes isolated from the re-fed rats and lowest in those from the starved rats. With time in culture, the activity of all these enzymes increased severalfold (2–5, depending on the enzyme under consideration) in hepatocytes isolated from fed and starved rats, whereas there was a severalfold (2–5) decrease in the activity of these enzymes in hepatocytes isolated from re-fed rats. The initial fatty acid content of the hepatocytes from re-fed rats was 2–3 times that in the other two groups of hepatocytes. The fatty acid content seemed to increase in all three groups of hepatocytes during the 96 h in culture, but these apparent increases were not statistically significant.

INTRODUCTION

Liver is the major site for the synthesis of endogenous lipids, which are secreted into the blood as lipoproteins (Geelen *et al.*, 1980; Clark, 1984). Lipogenic activity of the liver is profoundly influenced by the nutritional status of the animal. The rate of lipid synthesis and the activity of lipogenic enzymes are decreased during starvation or consumption of a fat-rich diet, whereas re-feeding of a fat-free carbohydrate-rich diet to starved animals markedly enhances the rate of lipid synthesis and the activity of lipogenic enzymes (Geelen *et al.*, 1980; Herzberg, 1983; Wakil *et al.*, 1983; Clark, 1984). The freshly isolated hepatocytes preserve the diurnal variations in lipid synthesis as found in the intact animals (Gibbons *et al.*, 1984), and also show decreased lipid synthesis, which accompanies starvation (Caro & Amatruda, 1982). It is not known for how long the prior nutritional status of the animal influences the lipogenic activity of hepatocytes in culture. The specific objectives of the present study were to determine (1) the effect of prior nutritional status (fed, starved, re-fed) on the activity of lipogenic enzymes (acetyl-CoA carboxylase, fatty acid synthase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase) in hepatocytes maintained up to 96 h in culture and (2) changes in the fatty acid content of these cells with time in culture. Our results indicate that the nutritional status of the animal before hepatocyte isolation profoundly influences the activity of lipogenic enzymes and the fatty acid content of these cells up to 96 h in culture.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats were obtained from Bantin and Kingman. Waymouth's MB 752/1 and Swim's S-77 media were purchased from GIBCO. Dexamethasone sodium phosphate and crystalline pig insulin were kindly given by Dr. K. Bohra of Organon and Dr. W. W. Bromer of Eli Lilly and Co., respectively. NADP⁺, NADPH, acetyl-CoA, malonyl-CoA, tri-iodothyronine, bovine serum albumin (fraction V; essentially fatty acid-free) were bought from Sigma Chemical Co. Collagenase (type II) was obtained from Worthington Biochemicals. NaH¹⁴CO₃ was supplied by New England Nuclear. Standard reference fatty acids were obtained from Nu-Check Prep and Supelco. All organic solvents were obtained from Burdick and Jackson.

Animal preparation

Rats initially weighing 100–150 g were purchased and then maintained in individual cages with free access to laboratory chow and water. The ambient temperature of the animal quarters was 25 °C. The room was lighted between 06:00 and 18:00 h. The animals weighed 300–400 g at the time of liver perfusion, which was done between 08:00 and 09:00 h. Before liver perfusions, the rats were fed *ad libitum* ('Fed'), or starved for 24 h ('Starved'), or starved for 48 h and then re-fed for 48 h ('Re-fed') with a diet containing (w/w) sucrose (65%), glycerol (5%) and egg albumin (30%).

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Hepatocyte isolation and culture

Hepatocytes were isolated by the method of Berry & Friend (1969) as modified by Bonney *et al.* (1974), with additional modifications as previously reported (Kelley & Potter, 1978, 1979; Kelley & Kletzien, 1984). Hepatocytes were suspended (2×10^6 /ml) in Waymouth's MB 752/1 medium supplemented with insulin ($0.2 \mu\text{M}$), dexamethasone ($1 \mu\text{M}$), tri-iodothyronine ($1 \mu\text{M}$), penicillin (100 units/ml) streptomycin ($100 \mu\text{g/ml}$), gentamycin ($20 \mu\text{g/ml}$) and defatted bovine serum albumin, fraction V (0.5 mg/ml). The cell suspension was inoculated (6 ml/dish) in 100 mm-diam. collagen-coated culture dishes, which were maintained at 37°C in an atmosphere of air/ CO_2 (19:1). Medium was initially changed at 3 h after plating and then every 24 h. At the specified times as shown in the Results section, the Waymouth's medium was removed by aspiration, and cells were washed four times with ice-cold phosphate-buffered saline or Hanks' Hepes buffered salt solution and then maintained frozen at -80°C until further processed.

Enzyme analysis

Culture dishes stored at -80°C were removed from the freezer and placed on ice. Cells from each dish were scraped with 1 ml of Hepes (0.2 M)/EDTA (0.1 mM)/2-mercaptoethanol (10 mM) buffer, pH 7.4. They were subjected to freezing and thawing three times and immediately centrifuged at $20000 g$ for 30 min at 4°C . The supernatant obtained after the above centrifugation was used to determine the enzymes and protein concentration. Results for enzymes are expressed as munits/mg of protein. Protein concentration was determined by the method of Lowry *et al.* (1951). Fatty acid synthase activity was determined spectrophotometrically by the method of Nepokroeff *et al.* (1975), in 0.5 M -potassium phosphate buffer, pH 7.0, containing malonyl-CoA ($200 \mu\text{M}$), acetyl-CoA ($66 \mu\text{M}$) and NADPH ($200 \mu\text{M}$). A unit of fatty acid synthase is taken as the amount of enzyme protein required to synthesize 1 nmol of palmitic acid (equivalent to the oxidation of 14 nmol of NADPH)/min at 30°C . Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities were also determined spectrophotometrically as previously reported (Kelley & Kletzien, 1984); 1 unit of either enzyme represents the amount of enzyme necessary for the production of $1 \mu\text{mol}$ of NADPH/min at 30°C . Acetyl-CoA carboxylase was determined as described by Inoue & Lowenstein (1975): $50 \mu\text{l}$ of the enzyme supernatant in Hepes buffer was added to $50 \mu\text{l}$ of the activation buffer [Tris/HCl (100 mM), sodium citrate (40 mM), MgCl_2 (40 mM), dithiothreitol (2 mM), bovine serum albumin (1 mg/ml), pH 7.5] and incubated in a shaking water bath at 37°C for 20 min. The reaction was started by adding 0.5 ml of the reaction mixture (final concentrations of reagents were: Tris/HCl 50 mM , acetyl-CoA 3 mM , ATP 1 mM , sodium citrate 20 mM , MgCl_2 20 mM , albumin 0.5 mg/ml , dithiothreitol 1 mM , NaHCO_3 20 mM , $\text{NaH}^{14}\text{CO}_3$ 0.2 Ci/mol), and allowed to proceed for 15 min, at which time the reaction was stopped by adding $100 \mu\text{l}$ of 4 M-HCl . The samples were dried under vacuum, hydrated, dissolved in Nuclear-Chicago Solubilizer (NCS) and mixed with Aqueous Counting Scintillant (ACS) for scintillation counting. One unit of acetyl-CoA carboxylase represents $1 \mu\text{mol}$ of malonyl-CoA formed/min at 37°C . Lipids were extracted

and analysed by the methods described by Nelson (1972, 1975).

RESULTS

Effect of nutritional status on the activity of lipogenic enzymes

The activities of the enzymes acetyl-CoA carboxylase, fatty acid synthase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in hepatocytes isolated from fed, starved and re-fed rats and then cultured up to 96 h are shown in Figs. 1–4 respectively. The initial activities of all these enzymes were severalfold higher in hepatocytes isolated from the re-fed rats than the corresponding activities in hepatocytes from fed animals, which in turn were higher than the corresponding activities in hepatocytes from starved animals. The differences in the initial activities of these enzymes among the three nutritional groups were in the sequence

Fig. 1. Acetyl-CoA carboxylase activity of cultured hepatocytes

Hepatocytes were isolated from the livers of rats continuously fed (○), starved for 24 h (△), or starved for 48 h and then re-fed with a carbohydrate-rich fat-free diet for 48 h (□), and then maintained in Waymouth's medium containing insulin ($0.2 \mu\text{M}$), dexamethasone ($1 \mu\text{M}$) and tri-iodothyronine ($1 \mu\text{M}$) throughout the culture period. Fresh medium was provided at 3, 24, 48 and 72 h after the initial plating. Acetyl-CoA carboxylase activity was determined in the supernatant prepared from the cells frozen at 3, 24, 48, 72 and 96 h after plating. Data shown are means $\pm$ S.D. for four to six culture dishes from a representative experiment for each nutritional group of rats. Standard deviations were not shown are within the symbols. The activity at 96 h in culture as percentage of that at 3 h for pooled data from all experiments was: re-fed group, 24 ± 8 ($n = 3$, $P < 0.001$); starved group, 215 ± 40 ($n = 5$, $P < 0.05$); fed group, 248 ± 59 ($n = 4$, $P < 0.1$). These data represent means $\pm$ S.E.M. for the numbers of experiments indicated. Statistical significance was estimated by using Student's two-tailed t distribution.

Fig. 2. Fatty acid synthase activity of cultured hepatocytes

Experimental details are described in the legend to Fig. 1. The activity at 96 h as a percentage of the initial enzyme activity was: re-fed group 24 ± 8 ($n = 3$, $P < 0.001$); starved group 238 ± 26 ($n = 5$, $P < 0.005$); fed group 277 ± 71 ($n = 4$, $P < 0.1$).

Fig. 3. Glucose-6-phosphate dehydrogenase activity of cultured hepatocytes

Experimental details are described in the legend to Fig. 1. The activity at 96 h as a percentage of the initial enzyme activity was: re-fed group 26 ± 6 ($n = 3$, $P < 0.001$); starved 498 ± 32 ($n = 5$, $P < 0.001$); fed group 378 ± 100 ($n = 4$, $P < 0.05$).

Fig. 4. 6-Phosphogluconate dehydrogenase activity of cultured hepatocytes

Experimental details are described in the legend to Fig. 1. The activity at 96 h as a percentage of the initial enzyme activity was: re-fed group 42 ± 2 ($n = 3$, $P < 0.001$); starved group 131 ± 13 ($n = 5$, $P < 0.1$); fed group 143 ± 19 ($n = 4$, $P < 0.1$).

glucose-6-phosphate dehydrogenase > fatty acid synthase > acetyl-CoA carboxylase > 6-phosphogluconate dehydrogenase. With increasing time in culture, the activities of all the four enzymes continued to decrease up to 96 h in hepatocytes from re-fed rats, whereas they all increased in hepatocytes from fed and starved rats. During the first 24 h in culture there was no detectable increase in the activity of any of these enzymes in hepatocytes from fed and starved rats, whereas there were marked decreases in the activities of all four enzymes in hepatocytes from the re-fed rats. The time taken in culture to reach maximum enzyme activity in hepatocytes from fed and starved rats varied for each enzyme, but it was generally around 72 h.

Cellular fatty acid content of cultured hepatocytes

The initial fatty acid contents in hepatocytes from starved, fed and re-fed animals were 111 ± 22 , 70 ± 10 and 227 ± 35 μg of fatty acid/mg of protein respectively (means $\pm$ S.E.M., $n = 6$). The fatty acid content in all three groups of hepatocytes appeared to increase during the 96 h culture period, but these apparent increases did not reach statistical significance (results not shown).

DISCUSSION

Our results reported here show that the nutritional status of the animal before the isolation of hepatocytes influenced the activity of lipogenic enzymes and the fatty acid content of these cells. Starvation for 24 h decreased the activity of all four enzymes, whereas re-feeding with a fat-free high-carbohydrate diet markedly increased the activity of all these enzymes (Figs. 1–4). The nutritional status of the animal before hepatocyte isolation seemed to have most dramatic effects on the activities of glucose-6-phosphate dehydrogenase and fatty acid synthase, but it had the lowest effect on the activity of 6-phosphogluconate dehydrogenase. Our results obtained with isolated hepatocytes regarding the effect of

nutritional status on the activity of lipogenic enzymes are in agreement with those previously obtained with intact animals (Tepperman & Tepperman, 1958; Potter & Ono, 1961; Allmann *et al.*, 1965; Gibson *et al.*, 1965; Yugari & Matsuda, 1967; Sassoon *et al.*, 1968; Burton *et al.*, 1969; Kelley *et al.*, 1975; Kim & Elson, 1981; Berke & Kaplan, 1983). The enzyme activities in hepatocytes cultured for 96 h seem to be controlled by the interaction between factors (hormonal and nutritional) to which the cells were exposed *in vivo* and those added to the culture medium.

All three groups of hepatocytes were maintained in a medium rich in glucose and supplemented with the lipogenic hormones, but these factors could stimulate the lipogenic enzymes only in hepatocytes from the fed and starved animals. In hepatocytes from the re-fed rats, the activity of lipogenic enzymes continued to decrease despite the presence of these stimulators of lipogenesis. It is possible that in the hepatocytes from the re-fed rats some inhibitory signals override the influence of the stimulatory signals for lipid synthesis, or some factor required to maintain enzyme activity *in vivo* is missing from the culture medium. The nature of these inhibitory signals is not clear, but free fatty acids and their CoA derivatives have been suggested (Eger-Neufeldt *et al.*, 1965; Taketa & Pogell, 1966).

The stimulation of glucose-6-phosphate dehydrogenase activity in hepatocytes isolated from fed and starved rats, as reported here, is consistent with our (Kelley & Kletzien, 1984) and other previous reports (Kurtz & Wells, 1981; Nakamura *et al.*, 1982); however, we are not aware of any reports where the stimulation of acetyl-CoA carboxylase and fatty acid synthase has been reported in the primary cultures of hepatocytes maintained in a serum-free medium. In hepatocytes isolated from meal-fed rats and then maintained in the absence of lipogenic hormones, the activity of acetyl-CoA carboxylase could be restored to the starting value by providing the lipogenic hormones to those cells maintained without hormones for 24 h, but the activity could not be increased above that found in the freshly isolated hepatocytes (Salati *et al.*, 1985). These differences may be due to the different nutritional status of the animal, the nutrient composition of the culture medium or the integrity of the cultured hepatocytes.

Our results indicate that cultured hepatocytes are a useful model system for describing the nutritional regulation of hepatic lipogenic activity.

We are most appreciative of the technical assistance provided by Ms. Claire Bunney and Ms. Perla C. Schmidt during the course of these studies.

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