Evidence for dissociation of gluconeogenesis stimulated by non-esterified fatty acids and changes in fructose 2,6-bisphosphate in cultured rat hepatocytes

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In order to examine the role of fructose 2,6-bisphosphate (Fru-2,6- P_2) in non-esterified-fatty-acid-stimulated gluconeogenesis, Fru-2,6- P_2 levels were measured in cultured rat hepatocytes under conditions mimicking the fasted state. After addition of either 1.5 mM-palmitate or 10 nM-glucagon, [14 C]lactate incorporation into glucose increased 2-fold, but only glucagon suppressed Fru-2,6- P_2 . Prevention of palmitate oxidation with a carnitine palmitoyltransferase-I inhibitor (2-bromopalmitate) diminished glucose production and Fru-2,6- P_2 levels. Addition of exogenous glucose to the media increased Fru-2,6- P_2 in a dose-related manner, which was further augmented by addition of palmitate. When Fru-2,6- P_2 levels were examined in cells cultured under conditions mimicking the fed state (significantly higher basal Fru-2,6- P_2 levels and lower glucose production), palmitate oxidation was associated with a significant fall in Fru-2,6- P_2 . In conclusion, the present studies have demonstrated a dissociation between fatty-acid-stimulated gluconeogenesis and changes in Fru-2,6- P_2 in cultured rat hepatocytes. Further experiments suggest that the accumulation of intracellular hexose 6-phosphate as a result of fatty-acid-stimulated gluconeogenesis masks a putative inhibitory effect of fatty acids on Fru-2,6- P_2 concentrations.

INTRODUCTION

It is widely recognized that non-esterified fatty acids stimulate hepatic gluconeogenesis [1–4] and that prevention of mitochondrial β -oxidation of long-chain fatty acids with carnitine palmitoyltransferase-I (CPT-I) inhibitors leads to a consistent decrease in fatty-acid-stimulated gluconeogenesis [5,6]. A key regulatory step in gluconeogenesis is the dephosphorylation of fructose 1,6-bisphosphate. The accumulation of citrate during fatty acid oxidation is known to decrease the activity of phosphofructokinase-1 (PFK-1) and enhance activity of fructose-1,6-bisphosphatase (Fru-1,6- P_2 ase) [7]. Both of these effects are potentiated by decreased levels of fructose 2,6-bisphosphate (Fru-2,6- P_2) (reviewed in [8]). Changes in hepatocellular levels of Fru-2,6- P_2 after administration of insulin (increased) or glucagon (decreased) support a central role for Fru-2,6- P_2 in carbohydrate metabolism [8,9]. In the fed state, high insulin levels and low glucagon levels result in elevated Fru-2,6- P_2 concentrations and decreased rates of gluconeogenesis. On the other hand, in the fasted state, when the insulin/glucagon ratio is low and lipid oxidation predominates, Fru-2,6- P_2 levels are decreased and gluconeogenesis predominates. Although it has been suggested that fatty acid oxidation lowers Fru-2,6- P_2 concentrations by inhibiting phosphofructokinase-2 (PFK-2) [10], these experiments were carried out at unphysiological glucose concentrations. No studies to date have demonstrated a significant fatty-acid-mediated decrease in Fru-2,6- P_2 in the absence of marked hyperglycaemia. The present studies were performed to determine the role of Fru-2,6- P_2 in fatty-acid-induced gluconeogenesis, using primary cultured hepatocytes.

EXPERIMENTAL

Materials

L-[U- 14 C]Lactate (89 mCi/mmol) was obtained from ICN,

Irvine, CA, U.S.A. (ethanol present in the tracer in small quantities was removed by evaporation under nitrogen before use). Insulin and glucagon were generously provided by Eli Lilly and Co., Indianapolis, IN, U.S.A. Enzymic assays were performed with enzyme preparations obtained from Boehringer-Mannheim, Indianapolis, IN, U.S.A. All other chemicals were obtained from Sigma, St. Louis, MO, U.S.A.

Preparation of cultured hepatocytes

Male Sprague-Dawley rats weighing 200–250 g were anaesthetized with ether, and the liver was perfused with 0.5% collagenase, which reduces the organ to a suspension of mostly single cells. Parenchymal cells were suspended in culture medium after separation from debris and other cell types by low-speed centrifugation. Batches of the isolated cells containing 3.5×10^6 cells in a total volume of 3 ml were plated without delay in 60 mm $\times$ 15 mm plastic culture dishes previously coated with Matrigel [11]. The latter matrix has replaced the use of calf skin collagen in our laboratory because of enhanced viability and greater retention of adult hepatocyte function. The plates were swirled carefully to ensure even distribution of cells over the surface of the plate and were then incubated under air/CO₂ (19:1) at 37 °C. Within 4 h, the cells had attached to the Matrigel surface, and by 12–24 h they had formed a confluent monolayer. Culture medium was changed every 24 h and consisted of Eagle's minimal essential medium vitamin mixture plus amino acids and salts of Hanks' medium 199, with the following modifications: ornithine (20 mg/l) was substituted for arginine, hydroxyproline and Fe(NO₃)₃ were omitted, and NaHCO₃ was added at a concentration of 1.26 g/l. Dexamethasone (10 μ M [12]) was also added to the culture medium to enhance cell viability, and because of its well-described effects on the maintenance of Fru-2,6- P_2 ase/PFK-2 mRNA [13]. In order to examine potential differences in gluconeogenesis, cells were cultured as described by

Abbreviations used: Fru-2,6- P_2 , fructose 2,6-bisphosphate; PFK-1, 6-phosphofructo-1-kinase; PFK-2, 6-phosphofructo-2-kinase; Fru-2,6- P_2 ase, fructose-2,6-bisphosphatase; Fru-1,6- P_2 ase, fructose-1,6-bisphosphatase; CPT-I, carnitine palmitoyltransferase-I.

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Probst *et al.* [14]. During the initial 24 h after isolation, hepatocytes were grown in the presence of 0.5 nM-insulin. Half of the plates were incubated for an additional 48 h with 10 nM-insulin, and the remaining plates were incubated in media containing 10 nM-glucagon and 0.5 nM-insulin for the same 48 h period. (In previous experiments comparing glucagon- and insulin-cultured cells in this laboratory, a shorter (24 h) period of differential culture was used, and cells were cultured on calf skin collagen instead of Matrigel [15].) Each perfusion routinely yielded 80–110 plates of cultured cells (3.5×10^6 cells/plate) from a single rat liver.

Evidence of sustained viability of hepatocytes in cultures has been provided by reports from this laboratory and elsewhere [15,16]. All cultures used in these experiments were > 90% viable, judged by the criterion of Trypan Blue exclusion.

Experimental incubation

Experiments were performed on 72 h-cultured cells as previously described [15]. The culture medium was replaced by Hanks' salts containing, in addition, 25 mM-Tricine, pH 7.4, 5 mM-glucose, 1 mM-lactate and 5% (w/v) BSA (essentially fatty-acid-free). Fatty acids (palmitate or octanoate) were added to media as the salt in the concentrations described below. The fatty acid concentration was verified by g.l.c. (Preliminary experiments in which the fatty acid was solubilized with NH_4OH and then neutralized resulted in a suppression of gluconeogenesis which proved to be an effect of increased osmolarity.) Media were supplemented with 25000 d.p.m. of $[\text{U-}^{14}\text{C}]$ lactate. Evidence for steady-state conditions was obtained during preliminary time-course experiments which demonstrated linear incorporation of label into glucose in both the absence and the presence of fatty acid over a 3 h time period. All subsequent experiments were performed for 2 h. Media removed from the culture dishes at the completion of the 2 h incubation were placed on ice for the determination of glucose and ketone bodies. Samples (0.1 ml) of media were placed on sequential anion- (Dowex-1, Cl^- form) and cation- (Dowex-50, H^+ form) exchange columns, and the glucose was eluted in the neutral fraction. Eluates were collected into scintillation vials, to which scintillation fluid was added. All samples were counted for radioactivity in a Beckman LS 1700 liquid-scintillation counter with correction for quench. Additional samples of the media were assayed for β -hydroxybutyrate by a microfluorometric assay [17].

For determination of Fru-2,6- P_2 , cells were immediately scraped into 50 mM-NaOH and heated at 80 °C for 10 min. The samples were centrifuged at 3000 rev./min for 10 min and the

resulting supernatant was neutralized with 25 mM-acetic acid/0.1 M-Hepes. Samples of the supernatant were assayed for Fru-2,6- P_2 by the method of Van Schaftingen [18]. In separate experiments, cells were scraped into Hepes buffer and used to assess synthesis of new glycogen as previously described [19]. Total DNA was determined fluorimetrically after cell solubilization [20], and was approx. 30 μg /plate.

Each experiment was performed on sister plates of hepatocytes obtained from a single perfusion and was performed in triplicate. The average result of the three plates was taken as a single observation. Calculation of lactate incorporation into glucose is based on unlabelled lactate concentration (1 $\mu\text{mol}/\text{ml}$) and percentage incorporation of $[\text{U-}^{14}\text{C}]$ lactate into glucose. It was assumed that two lactate molecules are required for one glucose molecule. All results are expressed as means $\pm$ S.E.M. Comparison of groups was performed by Student's *t*-test.

RESULTS

The effects of 1.5 mM-palmitate on $[\text{U-}^{14}\text{C}]$ lactate incorporation into glucose and levels of Fru-2,6- P_2 were compared with those of glucagon, a hormone with well-characterized effects on hepatic glucose production and on the bifunctional enzyme PFK-2/Fru-2,6- P_2 ase, which determines Fru-2,6- P_2 concentrations [21]. Both palmitate and glucagon produced significant increases in glucose production, but only glucagon decreased levels of Fru-2,6- P_2 (Table 1). From previous investigations [10], a fatty-acid-induced fall in Fru-2,6- P_2 had been expected. Since a putative effect of palmitate on Fru-2,6- P_2 would likely have been due to fatty acid oxidation, a more readily oxidized fatty acid not requiring CPT-I for transport into the mitochondria (octanoate) was compared with palmitate. The dose-response curves in Fig. 1 indicate that octanoate was oxidized at a 4-fold greater rate than palmitate, as reflected by β -hydroxybutyrate production. Some of this difference may be explained by the absence of carnitine from the incubation media (results not shown). However, Fru-2,6- P_2 measurements varied minimally from baseline throughout the dose-response curve for both fatty acids (bottom panel), despite the differences in fatty acid oxi-

Table 1. Effect of 1.5 mM-palmitate or 10 nM-glucagon on lactate incorporation into glucose ($\mu\text{mol}/\text{h}$ per mg of DNA) and intracellular levels of Fru-2,6- P_2 (nmol/mg of DNA) in cultured rat hepatocytes

Cells were cultured for 48 h in serum-free media containing 1 nM-dexamethasone, 10 nM-glucagon and 0.5 nM-insulin, washed and incubated for 2 h in media containing 25000 d.p.m. of $[\text{U-}^{14}\text{C}]$ lactate with 1 mM-lactate, 5 mM-glucose and 5% BSA in the absence or presence of fatty acid or glucagon. Each experiment was performed in triplicate, and results are expressed as means $\pm$ S.E.M.: **P* < 0.001 compared with BSA control.

	Glucose production ($\mu\text{mol}/\text{h}$ per mg of DNA)	Fru-2,6- P_2 (nmol/mg of DNA)
BSA (<i>n</i> = 16)	0.68 $\pm$ 0.05	2.49 $\pm$ 0.23
Palmitate (<i>n</i> = 16)	1.40 $\pm$ 0.12*	2.08 $\pm$ 0.22
Glucagon (<i>n</i> = 9)	1.57 $\pm$ 0.12*	0.53 $\pm$ 0.10*

Fig. 1. Effect of increasing concentrations of palmitate (●) or octanoate (○) on lactate incorporation into glucose (top panel), β -hydroxybutyrate production (middle panel) and intracellular levels of Fru-2,6- P_2 (bottom panel) in rat hepatocytes

Results are expressed as means $\pm$ S.E.M. (*n* = 4).

Table 2. Effect of 1.5 mM-palmitate and/or 2 mM-2-bromopalmitate (BPA) on lactate incorporation into glucose ($\mu\text{mol/h}$ per mg of DNA), intracellular levels of Fru-2,6- P_2 (nmol/mg of DNA) and β -hydroxybutyrate (BOHB; $\mu\text{mol/h}$ per mg of DNA) levels in cultured rat hepatocytes

Conditions are as described in Table 1. Results are expressed as means $\pm$ S.E.M. ($n = 7$): ^a $P < 0.01$, ^b $P < 0.05$ compared with BSA.

	Glucose production	Fru-2,6- P_2	BOHB
BSA	0.68 ± 0.05	1.9 ± 0.5	1.04 ± 0.09
Palmitate	1.23 ± 0.11^a	1.7 ± 0.5	4.57 ± 0.57^a
BPA	0.54 ± 0.04	1.4 ± 0.2	0.57 ± 0.08^b
Palmitate + BPA	0.52 ± 0.03	1.1 ± 0.2	0.77 ± 0.05

Table 3. Effect of increasing media concentrations of glucose on intracellular levels of Fru-2,6- P_2 in cultured hepatocytes

Conditions are the same as those described in Table 1. Results are expressed as means $\pm$ S.E.M. ($n = 4$): ^{*} $P < 0.05$ compared with control.

Experimental conditions	Fru-2,6- P_2 (nmol/mg of DNA)
Glucose in medium (mM)	
0	1.1 ± 0.2
2.5	1.4 ± 0.1
5.0	$1.9 \pm 0.2^*$
10.0	$2.7 \pm 0.4^*$
1.5 mM-palmitate	
+ 5.0 mM-glucose	1.8 ± 0.1
+ 10.0 mM-glucose	2.2 ± 0.1
10 nM-Glucagon	
+ 5.0 mM-glucose	0.4 ± 0.1
+ 10.0 mM-glucose	0.4 ± 0.1

Table 4. Effect of 5% BSA or BSA and 1.5 mM-palmitate on lactate incorporation into glucose and intracellular levels of Fru-2,6- P_2 in rat hepatocytes cultured for 48 h in the presence of 10 nM-insulin or 10 nM-glucagon and 0.5 nM-insulin

Results are expressed as means $\pm$ S.E.M. ($n = 9$): ^a $P < 0.01$ compared with control; ^b $P < 0.01$ compared with hepatocytes cultured in insulin alone.

	Glucose production ($\mu\text{mol/h}$ per mg of DNA)	Fru-2,6- P_2 (nmol/mg of DNA)
Insulin (10 nM)		
BSA	0.35 ± 0.04	4.34 ± 0.29
Palmitate	0.59 ± 0.08^a	3.38 ± 0.26^a
Glucagon + insulin		
BSA	0.63 ± 0.05^b	2.44 ± 0.34^b
Palmitate	1.35 ± 0.15^a	2.08 ± 0.34

dation. Gluconeogenesis (top panel) reached a peak at the dose of the respective fatty acid giving a 2-fold rise in β -hydroxybutyrate. Further fatty acid oxidation above that amount (octanoate experiments) did not enhance [U - ^{14}C]lactate incorporation into glucose.

To examine the role of endogenous fatty acid oxidation on changes in Fru-2,6- P_2 , the CPT-I inhibitor 2-bromopalmitate was used [22]. When cells were exposed to 1.5 mM-palmitate, lactate incorporation into glucose increased 2-fold (Table 2), and β -hydroxybutyrate production also increased. However, both

effects were almost completely inhibited by addition of 2-bromopalmitate. Lactate incorporation into glucose fell when 2-bromopalmitate was added to the control incubation media, which paralleled a fall in β -hydroxybutyrate production. In addition, the inhibition of endogenous fatty acid oxidation with 2-bromopalmitate failed to increase Fru-2,6- P_2 levels; in fact, Fru-2,6- P_2 levels tended to be decreased, although not significantly. When cells were incubated in the presence of both 2-bromopalmitate and 1.5 mM-palmitate, a further fall in Fru-2,6- P_2 was observed, without an effect on glucose or ketone production.

In order to examine the possibility that increased concentrations of hexose 6-phosphates derived from gluconeogenesis may have obscured the fall in Fru-2,6- P_2 expected from fatty acid oxidation, the effect of changes in exogenous glucose availability was studied. When increasing concentrations of glucose (0–10 mM) were added to the incubation media, intracellular levels of Fru-2,6- P_2 increased (Table 3). A similar rise in Fru-2,6- P_2 with glucose was seen in the presence of palmitate when the exogenous glucose concentration was increased from 5 to 10 mM. However, the low Fru-2,6- P_2 concentrations in glucagon-treated cells did not respond to increasing glucose availability. In the latter case, it is possible that any stimulatory effect of intracellular hexose 6-phosphate to raise levels of Fru-2,6- P_2 would have been over-ridden by the potent cyclic-AMP-mediated phosphorylation of the bifunctional enzyme [21], an effect not shared by palmitate.

The availability of cells cultured in insulin alone provided an opportunity to test further the hypothesis that accelerated gluconeogenesis with enhanced hexose 6-phosphate accumulation in the cells studied thus far may have masked an inhibitory effect of fatty acid oxidation on Fru-2,6- P_2 levels. Hepatocytes cultured for 48 h in the presence of insulin (10 nM), but not glucagon, are known to be less gluconeogenic than cells cultured with both insulin (0.5 mM) and glucagon [14]. As shown in Table 4, the incorporation of lactate carbon into glucose was significantly less in insulin-cultured cells than in cells cultured with glucagon and insulin. The diminished incorporation of labelled lactate into glucose in the insulin-cultured cells compared with the glucagon-cultured cells was associated with greater basal levels of Fru-2,6- P_2 . After addition of 1.5 mM-palmitate, a significant increase in gluconeogenesis was observed in both groups, albeit much greater in the latter cells. However, the addition of palmitate decreased Fru-2,6- P_2 levels significantly in the insulin-cultured cells, but not in the glucagon-cultured cells.

DISCUSSION

Glucagon and fatty acids are both potent stimulators of gluconeogenesis. Since Fru-2,6- P_2 is alleged to be an important mediator of gluconeogenesis, it was surprising to observe that fatty acids, unlike glucagon, failed to decrease Fru-2,6- P_2 levels. Glucagon decreases Fru-2,6- P_2 by a cyclic-AMP-mediated phosphorylation of PFK-2/Fru-2,6- P_2 ase [21]. In the single report indicating an inhibitory effect of fatty acids on Fru-2,6- P_2 , it was suggested that an inhibitory metabolite of fatty acid oxidation (citrate) inhibits PFK-2 as it does PFK-1 [10]. However, it should be pointed out that these studies were performed at supraphysiological glucose concentrations (> 20 mM) in order to stimulate glycolysis. Only in cells which have a low gluconeogenic potential could a modest inhibitory effect on Fru-2,6- P_2 concentration be observed in the present studies. In hepatocytes with a greater gluconeogenic potential (glucagon-cultured), an inhibitory effect of palmitate on Fru-2,6- P_2 could not be observed. However, an increase in extracellular glucose concentration increased levels of Fru-2,6- P_2 in such hepatocytes, presumably by providing substrate for PFK-2 [23]. Moreover, the addition of

10 mM-glucose to cells exposed to palmitate increased Fru-2,6- P_2 despite continued fatty acid oxidation. These experiments suggest that the products of gluconeogenesis (hexose 6-phosphates) might mask the putative inhibitory effect of fatty acids on Fru-2,6- P_2 . Additional support for this explanation comes from experiments in which a fall in Fru-2,6- P_2 is observed when endogenous fatty acid oxidation (and glucose production) is inhibited with a CPT-I inhibitor. Comparison of the change in Fru-2,6- P_2 observed in hepatocytes cultured in the absence or presence of glucagon after exposure to palmitate (Table 4) also suggests that the greater the stimulation of glucose production, the less predictable is the fall in Fru-2,6- P_2 . Thus the present studies suggest that a threshold level of glucose 6-phosphate (and fructose 6-phosphate) sufficient to inhibit Fru-2,6- P_2 ase and stimulate PFK-2 would mask the inhibitory effect of fatty acid oxidation on Fru-2,6- P_2 .

Such a threshold has been suggested in studies examining hepatic glycogen storage. During glucose refeeding of starved rats, levels of Fru-2,6- P_2 remain suppressed for several hours, as glycogen storage occurs through the so-called indirect (gluconeogenic) pathway [24,25]. Thereafter, Fru-2,6- P_2 levels increase toward those observed in the fed state [25,26]. When intracellular glucose intermediates have been measured during refeeding, the rise in Fru-2,6- P_2 has been associated with increases in hexose 6-phosphate concentrations from fasting to fed levels [27]. Unfortunately, intracellular levels of hexose 6-phosphates could not be measured accurately in the small sample size available in our cultured hepatocytes.

Because the operation of the glucose-fatty acid cycle is dependent on active glucose utilization [7,10], our failure to demonstrate a suppressive effect of fatty acid oxidation on Fru-2,6- P_2 may be related to the minor contribution of carbohydrate as a metabolic fuel in cultured hepatocytes. Hue *et al.* [10] have reported significant glucose utilization in freshly isolated rat hepatocytes exposed to media glucose concentrations in excess of 20 mM. However, when examined at physiological glucose concentrations or when levels of Fru-2,6- P_2 are below 5 nmol/mg of DNA, hepatic glucose utilization is minimal [28]. We have also found glucose utilization (as measured by $^3\text{H}_2\text{O}$ production from $[2\text{-}^3\text{H}]\text{glucose}$ or $^{14}\text{CO}_2$ production from $[6\text{-}^{14}\text{C}]\text{glucose}$) to be a minor pathway (< 1%) in cultured hepatocytes which is not altered by the presence of exogenous fatty acids [29]. Thus decreased glucose utilization, which would be expected to enhance the accumulation of hexose 6-phosphates derived from gluconeogenesis, may have sustained Fru-2,6- P_2 levels during exposure to fatty acids.

Impaired glycogen storage could also significantly alter intracellular levels of glucose 6-phosphate. As long as the production of glucose 6-phosphate is matched by its incorporation into glycogen, levels of hexose 6-phosphates should not accumulate [27], and the expected fatty-acid-mediated fall in Fru-2,6- P_2 would be observed. In hepatocytes which have increased glycogen stores, persistent glycogenolysis (and perhaps elevated hexose 6-phosphate levels) has been postulated to explain a delay in the fall in Fru-2,6- P_2 after exposure to glucagon [30]. However, cultured rat hepatocytes store glycogen poorly in the absence of insulin [15]. In the present studies, a small (< 200 nmol/h per mg of DNA) accumulation of labelled glycogen was observed during 2 h of incubation which increased insignificantly after addition of palmitate to the media (results not shown). It is therefore likely that the failure of Fru-2,6- P_2 to decrease after fatty acid stimulation results in part from impaired disposal of hexose 6-phosphates derived from gluconeogenesis.

In conclusion, the present studies have demonstrated a dissociation between fatty-acid-stimulated gluconeogenesis and changes in Fru-2,6- P_2 in cultured rat hepatocytes. Further experiments suggest that the accumulation of intracellular hexose 6-phosphate as a result of fatty-acid-stimulated gluconeogenesis masks a putative inhibitory effect of fatty acid on Fru-2,6- P_2 concentrations.

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