Interactions Between Gluconeogenesis and Fatty Acid Oxidation in Isolated Sheep Hepatocytes

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ABSTRACT

The interaction of gluconeogenesis and fatty acid oxidation in isolated sheep hepatocytes was studied. Addition of tetradecylglycidic acid, an inhibitor of carnitine palmitoyltransferase I (EC 2.3.1.21), to isolated hepatocytes inhibited gluconeogenesis from a mixture of pyruvate plus lactate and from propionate alone. Inhibition constants for tetradecylglycidic acid on gluconeogenesis were 4.77 ± 1.00 μM and 7.25 ± 1.52 μM, respectively, for pyruvate plus lactate and for propionate as gluconeogenic substrates. The inhibition constants were not different. At the highest substrate concentrations examined, gluconeogenesis from pyruvate plus lactate and from propionate in the presence of 10 μM tetradecylglycidic acid was 47.3 and 41.4% of their respective controls. Similar to previous observations with butyrate, caproate addition inhibited gluconeogenesis from propionate by isolated hepatocytes and was unable to prevent inhibition of gluconeogenesis induced by tetradecylglycidic acid. Carnitine palmitoyltransferase I activity was lower in mitochondria isolated from hepatocytes preincubated with insulin than in control hepatocytes. The data suggest 1) that maximum rates of gluconeogenesis in isolated sheep hepatocytes from either pyruvate plus lactate or from propionate as gluconeogenic substrates require β-oxidation, 2) that intermediates common to the metabolism of butyrate and caproate may be involved in the inhibition of propionate conversion to glucose by isolated sheep hepatocytes, and 3) that carnitine palmitoyltransferase I activity in isolated sheep hepatocytes can be modulated by insulin treatment.

(Key words: gluconeogenesis, fatty acid metabolism, hepatocytes)

Abbreviation key: CoA = coenzyme A, CPT I = carnitine palmitoyltransferase I, EGTA = ethyleneglycol-bis(β-aminoethyl ether)tetraacetic acid, OAA = oxaloacetate, TDGA = tetradecylglycidic acid.

INTRODUCTION

Ruminants are in a state of continuous gluconeogenesis because the ruminal fermentation of dietary carbohydrate limits the amount of glucose available for intestinal absorption (25). However, depending upon the physiological state of the animal, the principle gluconeogenic substrates utilized may change (5). Thus, propionate is the major source for glucose carbon in fed ruminants, but lactate and gluconeogenic amino acids predominate in ruminants deprived of feed. Previous research in sheep and several nonruminant species has demonstrated the requirement for hepatic β-oxidation in a permissive role to support maximal gluconeogenic rates (7, 10, 21, 23, 24). Carnitine palmitoyltransferase I (CPT I; EC 2.3.1.21) is the principle regulatory site for mitochondrial β-oxidation (17). Various studies utilizing tetradecylglycidic acid (TDGA), which inhibits CPT I activity and, consequently, inhibits long-chain fatty acid oxidation, have confirmed this relationship between β-oxidation and gluconeogenesis (23, 24). These studies, however, have utilized pyruvate or lactate as the major gluconeogenic substrates and have not addressed the ability of TDGA to influence gluconeogenesis from pro-
pionate. In addition, little work has been done to address the potential regulation of CPT I activity itself in ruminant liver.

Addition of octanoate to liver preparations in the presence of TDGA has been used to modulate β-oxidation rates. Octanoate addition has overcome TDGA-induced inhibition of gluconeogenesis; octanoate itself has no effect on gluconeogenic rates in nonruminant liver (23, 24). Results were similar with isolated sheep hepatocytes (7). In isolated goat and calf hepatocytes, octanoate addition also had no effect on rates of gluconeogenesis from propionate (2). In contrast to octanoate, butyrate—a short-chain fatty acid activated, as is octanoate, within the mitochondrial matrix—not only was ineffective in preventing TDGA-induced inhibition of gluconeogenesis but also itself inhibited gluconeogenesis in ruminant hepatocytes (1, 3, 4, 7). Inhibition of gluconeogenesis by butyrate was particularly noticeable when propionate was the gluconeogenic substrate. Valerate and isovalerate reportedly had no effect on rates of gluconeogenesis from propionate (2). The effect of caproate, a fatty acid intermediate to butyrate and octanoate in length, on hepatic gluconeogenesis from propionate has not yet been examined.

The objectives of this study were 1) to determine the effect of TDGA addition on gluconeogenesis by isolated sheep hepatocytes using either a mixture of pyruvate plus lactate, or propionate alone, as gluconeogenic substrates, 2) to determine whether caproate addition could inhibit propionate conversion to glucose (4), and 3) to determine whether pretreatment of isolated sheep hepatocytes with insulin could result in decreased mitochondrial CPT I activity.

MATERIALS AND METHODS

Materials

Tetradecylglycidic acid (McN-3802-21-98) was a generous gift from The R. W. Johnson Pharmaceutical Research Institute (Spring House, PA). Type I collagenase was obtained from Worthington Biochemicals, Inc. (Freehold, NJ). L-[Methyl-3H]carnitine was from Amersham, Inc. (Arlington Heights, IL). All other enzymes and reagents were from Sigma Chemical Co. (St. Louis, MO).

Methods

Hepatocyte Isolation. Hepatocytes were isolated from the hepatic caudate lobe of 12- to 32-wk-old fed Dorset ram lambs. The procedure was essentially as described previously (7). Briefly, the caudate lobe was removed immediately after slaughter and perfused through the major blood vessels with warm (37°C) calcium-free perfusion buffer [10 mM HEPES, pH 7.4, 140 mM NaCl, 7.1 mM KCl, .1 mM ethyleneglycol-bis(β-aminoethyl ether)tetraacetic acid (EGTA)]. Following transport to the laboratory, a recirculating perfusion of collagenase solution (10 mM HEPES, pH 7.4, 140 mM NaCl, 7.1 mM KCl, 5 mM CaCl2, and .25 g/100 ml Type 1 collagenase) at 37°C was begun. Hepatocytes were washed three times by centrifugation at 60 × g, followed by resuspension in ice-cold wash buffer (same as perfusion buffer but without EGTA). After the final wash, hepatocytes were resuspended to an approximate final volume for counting and viability estimates by trypan blue staining. The final volume of the cell suspension was adjusted based on cell counts to yield between 10 and 20 mg of viable cell dry weight/ml. Cell dry weights were determined by drying aliquots of hepatocytes and correcting for the salt content of an equal volume of wash buffer. Viable cell dry weights were calculated by multiplying total cell dry weights by the percentage of cell viability as determined by trypan blue dye exclusion.

Hepatocyte Incubations. Hepatocyte incubations were initiated by the addition of .5 ml of hepatocyte suspension, containing 5 to 10 mg of viable dry cell weight, to 2.5 ml of medium in 25-ml Erlenmeyer flasks. Medium was composed of Krebs-Ringer bicarbonate buffer supplemented with 25 mM HEPES, pH 7.4, 2 mM dl-carnitine, .2 mM dibutyryl cAMP (cyclic AMP), and .25 mM bovine serum albumin, and containing fatty acids and gluconeogenic substrates (propionate, pyruvate, lactate) at concentrations noted in the text. Pyruvate and lactate were added together as gluconeogenic substrate in a 1:10 ratio of pyruvate to lactate. Tetradecylglycidic acid was complexed with bovine serum albumin before addition to the incubation flasks. Solutions of TDGA were made fresh daily. Incubations were conducted at 37°C and were terminated by the addition of
concentrated perchloric acid. Media were neutralized with potassium carbonate and stored frozen until analyzed for glucose. Glucose was measured using a commercially available kit (kit 510, Sigma Chemical Co.). Results are expressed as nanomoles of net glucose produced per minute per milligram of viable dry cell weight, corrected for endogenous glucose production in the absence of added substrate.

Mitochondrial Isolation and CPT I Assay. Hepatocytes were incubated as described in the absence or presence of bovine pancreatic insulin (23.4 U/mg; .1 or .5 U/ml of final concentration). After 60 min, hepatocytes were collected by centrifugation at 60 × g, resuspended in a minimal volume of homogenization buffer (.25 M sucrose, 1 mM EDTA, 3 mM Tris, pH 7.4), and homogenized by six passes of the pestle in an ice-cold Dounce homogenizer (Wheaton Scientific Co., Millville, NJ). The homogenate was sequentially centrifuged for 10 min each at 500 × g and 5000 × g. The mitochondrial pellet was resuspended (.25 M sucrose, 3 mM Tris, pH 7.4), and the centrifugations were repeated. The pellet was resuspended to give a final mitochondrial protein concentration of 20 mg/ml, as determined using the Bradford dye-binding technique (Bio-Rad, Richmond, CA).

Carnitine palmitoyltransferase I activity was determined as reported by Saggerson and Carpenter (20), by following the incorporation of L-[methyl-3H]carnitine into palmitoyl-L-[methyl-3H]carnitine. Reactions were performed in a final volume of 1 ml and contained 30 μg of mitochondrial protein, 400 nmol of L-carnitine, 1 μCi of L-[methyl-3H]carnitine, and 80 nmol of palmitoylcoenzyme A (CoA). Results are expressed as nanomoles of palmitoyl-carnitine formed per minute per milligram of mitochondrial protein.

Statistical Analyses. Cell and mitochondrial incubations were conducted on three separate hepatocyte or mitochondrial preparations for each experiment with triplicate incubations for each treatment combination. Data were analyzed in a randomized complete block design, using the cell or mitochondrial preparations as blocks, with the treatment combinations present in a factorial arrangement. The significance of main effects was determined by analysis of variance, and specific treatment differences were determined by Fisher's least significant difference test (22). Inhibition constants for TDGA on gluconeogenesis from pyruvate plus lactate or from propionate were calculated from Lineweaver-Burk plots and compared by Student’s t test.

RESULTS AND DISCUSSION

Gluconeogenic rates in the present study were similar to those previously reported for isolated ruminant hepatocytes (1, 2, 3, 4, 7). Gluconeogenesis from either pyruvate plus lactate or from propionate was inhibited by 10 μM TDGA (Figures 1 and 2). At the highest substrate concentrations utilized, net glucose production from pyruvate plus lactate in the presence of 10 μM TDGA was 47.3% of control, whereas that from propionate was 41.4% of control. These results are similar to those obtained with isolated rat hepatocytes, for which 10 μM TDGA inhibited gluconeogenesis from either pyruvate or lactate by about 50% (24). Double reciprocal Lineweaver-Burk plots for gluconeogenesis from pyruvate plus lactate and from propionate were constructed (Figures 3 and 4). Linear regression equations for the Lineweaver-Burk plot of gluconeogenic rates from pyruvate plus lactate (Figure 3) were Y = (14.0 ± 1.4)X + (2.10 ± .29) and Y = (33.54 ± 9.95)X + (6.50 ± 2.08) for control and TDGA-treated sheep hepatocytes, respectively. The slopes of these lines were different (P < .05), and the lines appear to converge near a common X-intercept (Figure 3). This plot is similar to that expected for a noncompetitive inhibitor (9). The inhibition constant for TDGA on glucose production from pyruvate plus lactate from three experiments was 4.77 ± 1.00 μM (9). Regression equations for the double reciprocal plot of gluconeogenic rates with propionate as substrate (Figure 4) were Y = (.811 ± .096)X + (.993 ± .110) and Y = (.920 ± .375)X + (2.53 ± .43) for control and TDGA-treated sheep hepatocytes, respectively. The slopes of these lines were not different. The plot suggests an uncompetitive mode of inhibition for TDGA on gluconeogenesis from propionate (9). The inhibition constant for TDGA on glucose production from propionate from three experiments was 7.25 ± 1.52 μM (9), which was not different from that for pyruvate plus lactate. Thus, 10 μM TDGA
Inhibited gluconeogenesis from either pyruvate plus lactate or from propionate to a similar extent but appeared to do so via different mechanisms, as reflected in the Lineweaver-Burk plots.

As previously observed, addition of either acetate or octanoate had no effect on glucose production from propionate by isolated sheep hepatocytes (Figure 5). Acetate addition in the presence of TDGA had no effect on the ability of TDGA to inhibit gluconeogenesis from propionate. The presence of octanoate, however, prevented TDGA from inhibiting propionate conversion to glucose. Butyrate addition, however, inhibited gluconeogenesis from propionate by isolated sheep hepatocytes, reducing gluconeogenic rates to 61% of control (P < .05). Addition of butyrate had no effect on TDGA-induced inhibition of glucose production from propionate. Results were similar with the addition of caproate. Caproate proved to be as potent an inhibitor of gluconeogenesis from propionate as was butyrate. Caproate addition resulted in a reduction of gluconeogenic rates to 56.8% of control (P < .05). As for butyrate, caproate addition had no effect on TDGA-induced inhibition of propionate conversion to glucose.

Ruminants are in a state of continuous gluconeogenesis in part because of the exposure of the liver to relatively low insulin and high glucagon concentrations in the blood (16). Exposure of isolated sheep hepatocytes to insulin would thus be expected to decrease CPT I activity. Treatment of isolated sheep hepatocytes with insulin for 60 min prior to mitochondrial isolation from the hepatocytes resulted in a significant decrease in CPT I activity (Figure 6). Addition of .1 U/ml of insulin reduced CPT I activity to 83.9% of control (P < .05), whereas preincubation with .5 U/ml of insulin resulted in 77.1% of the control CPT I activity (P < .05). No difference in CPT I activity was observed between the two insulin concentrations tested.

Oxaloacetate (OAA) is a common intermediate in the conversion of pyruvate, lactate, and propionate carbon into glucose. However, pyruvate carbon and, consequently, lactate carbon are converted directly into OAA by the pyruvate carboxylase reaction, propionate carbon is converted to OAA subsequent to entry into the Krebs cycle as succinyl-CoA. Pyruvate carboxylase is a mitochondrial enzyme that requires acetyl-CoA as an activator for normal activity (6). The principle mechanism by which TDGA is thought to inhibit gluconeogenesis...
Figure 3. Lineweaver-Burk plot of glucose production by isolated sheep hepatocytes from pyruvate plus lactate in the presence and absence of tetradecylglycidate (TDGA). Double reciprocal plots were constructed of net glucose production rates and substrate concentrations (pyruvate plus lactate) using the mean values from Figure 1. Linear regression lines were $Y = (14.0 \pm 1.40)X + (2.10 \pm .29)$, $r^2 = .981$ for control; and $Y = (33.5 \pm 9.95)X + (6.50 \pm 2.08)$, $r^2 = .850$ for TDGA-treated sheep hepatocytes. $V$ = Reaction velocity. Slopes for the regression equations were different ($P < .05$).

Glucogenesis is via inactivation of pyruvate carboxylase as a result of decreased intramitochondrial acetyl-CoA concentrations, because of, in turn, reduced rates of fatty acid $\beta$-oxidation (23). This theory is supported by the noncompetitive kinetics displayed by the TDGA-induced inhibition of gluconeogenesis from pyruvate plus lactate (Figure 3). Classically, noncompetitive inhibition is observed when inhibitor binding occurs at a site distinct from the active site on an enzyme (9). Binding of a noncompetitive inhibitor can occur to either the enzyme or the enzyme-substrate complex. In either case, product formation is limited. Similar kinetic behavior occurs upon removal of an activator, such as acetyl-CoA, which can also bind to either enzyme or enzyme-substrate complex, resulting in decreased rates of product formation.

Acetyl-CoA has not been reported to activate any of the reactions by which propionate carbon is converted to glucose. Despite that, TDGA was still capable of inhibiting gluconeogenesis from propionate, albeit by a mechanism different from that observed with pyruvate plus lactate (Figures 3 and 4). This suggests that some aspect of propionate metabolism is dependent upon products of fatty acid $\beta$-oxidation. Noncompetitive inhibition, as displayed by TDGA on gluconeogenesis from propionate, is classically associated with binding of inhibitor to enzyme-substrate complex (9). At present, conceptualization of an inhibitory mechanism with these kinetic properties is difficult. One possible explanation is that ATP availability for propionate activation to propionyl-CoA becomes limiting in the presence of TDGA. The likelihood that reducing equivalents become limiting in response to reduced $\beta$-oxidation can probably be discounted in light of recent findings that alteration of cellular redox potential had little effect on gluconeogenic rates in isolated goat hepatocytes (2).

Octanoate is known to overcome TDGA-induced inhibition of gluconeogenesis in nonruminant liver preparations as well as sheep hepatocytes (7, 23, 24). Octanoate is activated within the mitochondrial matrix, bypassing the CPT I reaction inhibited by TDGA, which is necessary for transport of long-chain fatty acids into the mitochondria. Butyrate and other short- and medium-chain fatty acids are, as is octanoate, activated within the mitochondrial matrix.

Figure 4. Lineweaver-Burk plot of glucose production by isolated sheep hepatocytes from propionate in the presence and absence of tetradecylglycidate (TDGA). Double reciprocal plots were constructed of net glucose production rates and substrate concentrations using the mean values from Figure 3. Linear regression equations were $Y = (.811 \pm .096)X + (.993 \pm .110)$, $r^2 = .973$ for the control; and $Y = (.920 \pm .375)X + (2.53 \pm .43)$, $r^2 = .751$, for TDGA-treated hepatocytes. $V$ = Reaction velocity. Slopes for the regression equations were not different ($P > .05$).
matrix. Consequently, those fatty acids would be thought to overcome TDGA-induced inhibition of gluconeogenesis in a manner similar to that of octanoate. However, both butyrate and caproate inhibited gluconeogenesis from propionate (Figure 5). The mechanism by which this inhibition occurs is not currently known. A likely site of action is the acyl-CoA synthetase, which activates propionate to propionyl-CoA. Ruminant liver contains a specific propionyl-CoA synthetase (19). This enzyme reportedly exhibits no activity with butyrate as substrate and low activity with caproate as substrate. However, no competition studies were conducted to assess potential inhibitory effects of either butyrate or caproate on propionate activation. The structural similarity among propionyl-, butyryl-, and caproyl-CoA would suggest the potential for competition of butyrate or caproate on propionate activation. Speculation has been raised that absorbed butyrate is converted to β-hydroxybutyrate by the rumen epithelium to prevent inhibition of hepatic gluconeogenesis by ruminally derived butyrate (4). Portal infusions of butyrate in vivo, however, had no effect on hepatic gluconeogenesis (18). Thus, the question remains as to whether or not the observed effects of butyrate and caproate on in vitro hepatic gluconeogenesis have in vivo physiological significance.

Regulation of CPT I activity is thought to occur predominantly via changes in cytoplasmic malonyl-CoA concentrations (17). However, various reports (8, 11, 12) suggested the ability of insulin and glucagon to modulate CPT I activity directly in nonruminant liver, presumably by reversible covalent modification of the enzyme protein. Although intrahepatic malonyl-CoA concentration serves as the principle regulator of CPT I activity, changes in circulating insulin and glucagon concentrations, as reflected by the insulin:glucagon ratio, can result in alterations in both inherent CPT I activity and in sensitivity of CPT I to malonyl-CoA inhibition (8, 12, 14, 15). Inherent changes in CPT I kinetic properties are currently thought to play a role as a broad spectrum of mechanisms for the integrated control of hepatic β-oxidation and ketogenesis (26). Despite the fact that ruminant liver is not a major site for de novo fatty acid synthesis (13), the malonyl-CoA content of ruminant liver is comparable with that of rat liver and reportedly decreases significantly upon fasting (26), suggesting a role for...
malonyl-CoA in the regulation of hepatic CPT I activity and β-oxidation in ruminants. That sheep liver CPT I activity can decrease in response to in vitro insulin treatment suggests a further similarity in metabolic regulatory mechanisms between ruminant and nonruminant liver. Measurements of CPT I activity in hepatic mitochondria isolated from ruminants in a variety of metabolic states will need to be conducted to determine whether CPT I activities change in vivo in response to altered metabolic status.

REFERENCES


