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THE CHARACTERIZATION OF PERFLUOROSUCCINATE AS AN INHIBITOR OF GLUCONEOGENESIS IN ISOLATED RAT HEPATOCYTES

ROLAND B. GREGORY and MICHAEL N. BERRY*

Department of Medical Biochemistry, School of Medicine, Flinders University of South Australia, Bedford Park, South Australia 5042, Australia

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Abstract—The effects on metabolism of the fluorinated dicarboxylic acid, perfluorosuccinate, were examined in hepatocytes from fasted rats. Perfluorosuccinate (5 mM) inhibited gluconeogenesis from lactate by 80% and from pyruvate by 40%. Significant inhibition (up to 30%) occurred at a concentration of perfluorosuccinate of 50 μM. Cellular ATP levels were not affected by perfluorosuccinate, nor was the rate of formation of ketone bodies from palmitate, although the ratio [3-hydroxybutyrate]/[acetoacetate] was increased up to 5-fold relative to the control. An increased concentration of cellular L-malate was measured in the presence of perfluorosuccinate but this did not reflect inhibition of malate transport between the mitochondrial and cytoplasmic compartments. In addition, ethanol oxidation by hepatocytes was inhibited 25% by 1 mM perfluorosuccinate. Ureogenesis from ammonia was relatively insensitive to inhibition by perfluorosuccinate. In cytoplasmic extracts of rat liver, the activities of phosphoenolpyruvate carboxykinase and aspartate aminotransferase were inhibited 40–50% and 23%, respectively, by 1 mM perfluorosuccinate. The observed metabolic effects of perfluorosuccinate are consistent with inhibition of the activities of phosphoenolpyruvate carboxykinase and aspartate aminotransferase within the cytoplasm.

Metabolic inhibitors have long played an important role in the study of the regulation of cellular metabolism, particularly in relation to pathways such as gluconeogenesis, where preservation of the subcellular structure is necessary. One such class of inhibitors comprises the fluoro homologues of various substrates which normally form part of a metabolic pathway [1, 2]. The substitution of fluorine for hydrogen in carboxylic acid substrates causes little steric alteration in the structure of the molecule, thereby maximizing the recognition of the fluoro homologue by the appropriate enzymatic active centre. A wide range of fluorinated carboxylic or dicarboxylic acids has proved useful in the investigation of metabolic regulation [1, 3, 4]. In addition, the substrate and inhibitor effects of fluorinated succinic acids with both particulate and soluble succinate dehydrogenase, have been reported [5, 6]. In this paper we report the use of the fluorinated dicarboxylic acid, perfluorosuccinate, in rat hepatocytes, as a potent inhibitor of gluconeogenesis from lactate, and to a lesser degree, from pyruvate. The metabolic effects of perfluorosuccinate appear to be related to the inhibition of the activities within the cytoplasmic compartment of aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) and phosphoenolpyruvate carboxykinase (GTP:oxaloacetate carboxy-lysase, transphosphorylating, EC 4.1.1.32).

Part of this work has been presented at a meeting of the Australian Biochemical Society [7].

MATERIALS AND METHODS

Materials. Collagenase and enzymes for metabolite determination were from Boehringer Mannheim (F.R.G.) as was bovine serum albumin (fraction V), which was defatted by the method of Chen [8]. Palmitate, digitonin and antimycin were obtained from the Sigma Chemical Co. (St. Louis, MO). Palmitate was neutralized and dissolved in 0.15 M NaCl containing 9% bovine serum albumin. Quinolinic acid was from Matheson, Coleman and Bell (Ohio, U.S.A.), *n*-butylmalonic acid and FCCP† from Aldrich Chemical Co. (Milwaukee, WI) and perfluorosuccinic acid from Pfaltz and Bauer, Inc. (Conn., U.S.A.), (or from K&K Rare and Fine Chemicals, U.S.A.). [^{14}C]$NaHCO_3$, [^{14}C]ethanol and ACS II liquid scintillation fluid were purchased from Amersham International (Bucks, U.K.) and silicone oil from Dow Corning Corp. (Midland, MI). Other chemicals were of the highest quality commercially available.

Methods. Isolated hepatocytes from normal, male Hooded Wistar rats (250–300 g body mass), starved for 24 hr to deplete liver glycogen, were prepared according to a modification [9] of the method of Berry and Friend [10] and suspended in a medium of pH 7.4 with composition 137 mM NaCl, 5 mM KCl, 0.81 mM $MgSO_4$, 1 mM phosphate and 1 mM $CaCl_2$. The cells (90–120 mg wet wt.) were incubated at 37° in 2 ml of isotonic medium with the following composition: 146 mM Na^+, 5.4 mM K^+, 0.8 mM Mg^{2+}, 2.55 mM Ca^{2+}, 5.7 mM phosphate, 0.8 mM SO_4^{2-}, 25 mM HCO_3^-, 2.25% bovine serum albumin,

* Correspondence should be addressed to: Professor M. N. Berry.

† Abbreviations: FCCP, carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone; AST, aspartate aminotransferase (EC 2.6.1.1); PEPCK phosphoenolpyruvate carboxykinase (EC 4.1.1.32).

Table 1. A comparison of the effects of perfluorosuccinate and quinolinate on gluconeogenesis from lactate or pyruvate

	Glucose rate (μmol/min/g wet wt.)	[Malate] (μmol/g wet wt.)	B/A
2 mM Palmitate, 10 mM lactate, 1 mM pyruvate			
[Perfluorosuccinate], mM			
0	1.31 ± 0.04 (14)	1.18 ± 0.09 (12)	1.01 ± 0.05 (14)
0.05	0.87 ± 0.05 (6)	3.55 ± 0.20 (6)	1.99 ± 0.10 (6)
0.5	0.62 ± 0.05 (6)	5.62 ± 0.26 (6)	2.95 ± 0.12 (7)
5.0	0.27 ± 0.02 (10)	8.14 ± 0.35 (11)	5.07 ± 0.14 (9)
[Quinolinate], mM			
1	1.30 ± 0.02 (6)	1.33 ± 0.12 (6)	1.09 ± 0.11 (8)
5	0.84 ± 0.03 (7)	3.51 ± 0.38 (7)	2.49 ± 0.24 (8)
10	0.57 ± 0.03 (7)	5.26 ± 0.56 (7)	3.60 ± 0.33 (8)
2 mM Palmitate, 15 mM pyruvate			
[Perfluorosuccinate], mM			
0	0.95 ± 0.04 (9)	1.83 ± 0.14 (9)	0.88 ± 0.04 (9)
0.05	0.78 ± 0.05 (4)	2.96 ± 0.24 (4)	1.29 ± 0.08 (4)
0.5	0.70 ± 0.04 (4)	4.10 ± 0.29 (5)	1.64 ± 0.07 (5)
5.0	0.56 ± 0.03 (7)	5.17 ± 0.30 (7)	2.27 ± 0.08 (7)
[Quinolinate], mM			
1	0.92 ± 0.08 (4)	2.37 ± 0.31 (4)	0.95 ± 0.10 (4)
5	0.70 ± 0.07 (4)	3.42 ± 0.39 (4)	1.55 ± 0.16 (4)
10	0.57 ± 0.04 (4)	4.47 ± 0.34 (4)	2.01 ± 0.20 (4)

Hepatocytes were incubated for 35 min at 37° with 2 mM palmitate, 10 mM lactate and 1 mM pyruvate or 2 mM palmitate and 15 mM pyruvate. The inhibitors were present in the incubations as indicated. The data are shown as means ± SE with numbers of experiments in parenthesis. B/A = [3-hydroxybutyrate]/[acetoacetate].

and Cl^-. The concentration of Cl^- was varied between 100 and 115 mM to maintain isotonicity when the initial concentration of other anionic species was varied. The gas phase was 95% O_2, 5% CO_2. O_2-consumption in the presence of CO_2 was measured manometrically [11]. At the end of the incubation period (35 min), the incubation mixtures were deproteinized with an equal volume of 1 M perchloric acid and neutralized. Metabolites were measured by standard enzymatic techniques as described in [12] by means of a Cobas FARA automated analyser (Roche, Basle), the data being transferred to a PDP11/73 computer (D.E.C., U.S.A.) for subsequent processing. Separation of cytoplasmic and mitochondrial compartments was achieved by centrifuging a sample of digitonin-treated cells layered over silicone oil, according to [13].

Phosphoenolpyruvate carboxykinase activity was assayed by measuring the incorporation of $[^{14}C]HCO_3^-$ into malate according to the method of Chang and Lane [14] with GDP and dithiothreitol replacing IDP and glutathione, respectively. The activities of malate and lactate dehydrogenases, and of aspartate and alanine aminotransferases, were measured spectrophotometrically [12] at 30° in a Shimadzu UV-3000 spectrophotometer.

Rat liver mitochondria were isolated by differential centrifugation according to standard procedures [15] and the mitochondria resuspended to a protein concentration of about 60 mg/ml. Mitochondrial swelling was measured by following the decrease in extinction at 750 nm [16]. Alternatively, the rate of reduction of intra-mitochondrial NAD(P) upon addition of malate and either citrate or 2-oxoglutarate was measured at 340 nm in mitochondria oxidised and inhibited by the addition of both FCCP and antimycin [16].

RESULTS AND DISCUSSION

Inhibition of gluconeogenesis from lactate or pyruvate by perfluorosuccinate

Initial experiments with perfluorosuccinate demonstrated that when added to hepatocytes, it was a powerful inhibitor of gluconeogenesis from lactate, and to a lesser degree from pyruvate (Table 1). Palmitate was included in the incubations to promote greater rates of glucose synthesis although qualitatively similar results were obtained in its absence, the proportional degree of inhibition being the same. Pre-incubation of the cells with perfluorosuccinate was not required. The rate of accumulation of glucose was slightly bi-phasic with an increased rate of synthesis after 15–20 min. However, we consider that presenting average rates of gluconeogenesis over a 35 min incubation period does not introduce a significant error in interpretation of the results.

When hepatocytes were incubated in the presence of 2 mM palmitate, 10 mM lactate and 1 mM pyruvate, gluconeogenesis was inhibited by perfluorosuccinate, approximately 80% inhibition occurring at an inhibitor concentration of 5 mM (Table 1). The control respiration rate of 5.0 μmol/min/g wet wt. cells, was decreased 21% by 5 mM perfluorosuccinate. Values of the ratio [3-hydroxybutyrate]/[acetoacetate] and the malate concentration reached maxima at approximately 15–20 min, but nevertheless, their values at 35 min main-

Table 2. Comparison of the effects of perfluorosuccinate and quinolinate on hepatocytes metabolising ethanol and lactate

	Glucose rate	Ethanol rate	Lactate rate	[Malate]	B/A
[Perfluorosuccinate], mM					
0	0.35 ± 0.03 (13)	−1.39 ± 0.07 (10)	−1.38 ± 0.13 (10)	1.62 ± 0.13 (11)	2.42 ± 0.21 (11)
0.05	0.11 ± 0.02 (7)	−1.16 ± 0.05 (8)	−0.38 ± 0.04 (6)	2.07 ± 0.18 (8)	2.51 ± 0.28 (7)
0.10	0.08 ± 0.01 (6)	−1.11 ± 0.05 (7)	−0.37 ± 0.04 (5)	1.92 ± 0.15 (6)	2.29 ± 0.36 (7)
1.0	0.06 ± 0.02 (7)	−1.05 ± 0.02 (6)	−0.19 ± 0.04 (6)	2.06 ± 0.19 (7)	2.03 ± 0.17 (5)
[Quinolinate], mM					
1	0.25 ± 0.02 (4)	−1.35 ± 0.07 (5)	−0.73 ± 0.06 (3)	2.10 ± 0.25 (6)	3.31 ± 0.53 (5)
5	0.12 ± 0.01 (5)	−1.41 ± 0.06 (5)	−0.50 ± 0.03 (6)	4.76 ± 0.52 (7)	6.04 ± 0.44 (6)
10	0.05 ± 0.01 (5)	−1.28 ± 0.07 (5)	−0.50 ± 0.07 (5)	6.58 ± 0.13 (3)	6.16 ± 0.55 (4)

Hepatocytes were incubated for 35 min at 37° with 8 mM ethanol and 10 mM lactate. Perfluorosuccinate and quinolinate were present as indicated. The data are presented as means ± SE with the number of experiments in parenthesis. B/A = [3-hydroxybutyrate]/[acetoacetate]. Rates are expressed as μmol/min/g wet wt. cells and [malate] as μmol/g wet wt. cells.

tained an inverse relationship to the rate of gluconeogenesis (not shown). Cellular levels of 2-oxoglutarate and aspartate were increased approximately 60% when perfluorosuccinate was added, the increase occurring over the lower range of inhibitor concentration (<0.5 mM; data not shown). No significant changes occurred in the concentration of cellular ATP or in the rate of production of ketone bodies from palmitate (not shown).

For comparison, Table 1 contains data obtained with another gluconeogenic inhibitor, quinolinate, which inhibits the activity of PEPCK [17]. When lactate or pyruvate was the gluconeogenic substrate, perfluorosuccinate and quinolinate produced qualitatively similar effects (Table 1).

Inhibition of ethanol oxidation by perfluorosuccinate

Although the above suggested that perfluorosuccinate too, might be an inhibitor of PEPCK, perfluorosuccinate must have another (or additional) mode of action, in view of the different effects produced by it and quinolinate when added to cells metabolising ethanol and lactate (Table 2). Perfluorosuccinate inhibited both gluconeogenesis and ethanol oxidation without significantly changing the malate concentration and the ratio [3-hydroxybutyrate]/[acetoacetate]. A similar degree of inhibition of ethanol oxidation (namely 22% inhibition in the presence of 1 mM perfluorosuccinate) was observed when ethanol was the only added substrate. On the other hand, quinolinate had no significant effect on ethanol oxidation, but increased the [3-hydroxybutyrate]/[acetoacetate] ratio and malate concentration (Table 2). Perfluorosuccinate (up to a maximum concentration of 5 mM) did not inhibit ethanol removal (2.5 μmol/min/g wet wt. cells) when 15 mM pyruvate replaced 10 mM lactate in the incubation (not shown). Under these circumstances, reducing equivalents produced by the alcohol dehydrogenase reaction can be disposed of in the cytoplasm, leading to a quantitative reduction of pyruvate to lactate. This indicated that the inhibition of ethanol oxidation in hepatocytes is not due to inhibition of the activity of alcohol dehydrogenase (EC 1.1.1.1) *per se*, a finding in agreement with the observed absence of any significant inhibition by perfluorosuccinate (up to a maximum concentration of 5 mM) of the activity of the enzyme in digitonin-extracts of rat hepatocytes.

Shuttle requirements for gluconeogenesis and ethanol oxidation

As noted above, perfluorosuccinate inhibited the formation of glucose from lactate more strongly than from pyruvate. The principal difference between these pathways is the requirement for a malate–aspartate shuttle in the case of gluconeogenesis from lactate, for the transfer of carbon (as aspartate) to the cytosol, and a malate shuttle in the case of glucose synthesis from pyruvate, for the transport of carbon and hydrogen from the mitochondria to the cytoplasm [18–21]. Likewise, ethanol oxidation utilises the malate–aspartate and/or α-glycerophosphate shuttles to transfer reducing equivalents to the mitochondria [22, 23]. Since aspartate aminotransferase is an integral part of the malate–aspartate shuttle, it seemed possible that either this transferase or malate transport into the mitochondrion may be inhibited by perfluorosuccinate.

Intracellular malate concentration and malate transfer

Perfluorosuccinate caused large increases in malate concentration when cells were metabolising lactate or pyruvate, in the absence of ethanol (Table 1). Cellular fractionation studies revealed that these higher concentrations of malate reflected elevated levels of malate in both mitochondrial and cytoplasmic compartments qualitatively similar to those brought about by quinolinate inhibition (not shown). Malate is able to cross the mitochondrial membrane on several different carriers [24] and indeed, is an antiporter for the entry of 2-oxoglutarate and tricarboxylic acids such as citrate. In contrast to *n*-butylmalonate which inhibits the uptake of malate by isolated mitochondria [16], perfluorosuccinate did not inhibit the uptake of malate into mitochondria whether tested by the mitochondrial swelling method or by the method involving reduction of mitochondrial NAD(P) (not shown). Thus, it is improbable that the inhibition of gluconeogenesis from lactate or pyruvate in the presence of perfluorosuccinate is associated with any effects on malate

Table 3. Percentage inhibition of ureogenesis and gluconeogenesis

Inhibitor	% Inhibition of urea rate	% Inhibition of glucose rate
Perfluorosuccinate, mM		
0.1	5	44
1.0	16	58
5.0	22	72
10.0	36	82
Aminooxyacetate, mM		
0.50	84	90
Quinolinate, mM		
10	22	58

Hepatocytes were incubated at 37° for 35 min with 2 mM palmitate, 10 mM lactate, 1 mM pyruvate, 2 mM ornithine, 12 mM NH_4Cl and inhibitors as indicated. In the case of aminooxyacetate, cells were pre-incubated for 10 min with the inhibitor before addition of substrates. The uninhibited rates of ureogenesis and gluconeogenesis were 1.89 μmol/min/g wet wt. and 0.71 μmol/min/g wet wt., respectively. The results are from a representative experiment.

transport.

Enzyme kinetics with purified enzymes and extracts of hepatocytes

Investigations of the effect of perfluorosuccinate on pig-heart AST revealed competitive inhibition with respect to both 2-oxoglutarate ($K_i = 0.8$ mM) and aspartate ($K_i = 1.3$ mM). For comparison, the corresponding alanine aminotransferase was not inhibited by 0.8 mM perfluorosuccinate, nor was malate dehydrogenase, nor lactate dehydrogenase (rabbit muscle). This investigation was extended to rat liver extracts. In a cytosolic extract obtained by ultracentrifugation, the activities of alanine aminotransferase and malate dehydrogenase were not significantly changed when assayed in the presence of 1 mM perfluorosuccinate, whereas the activity of AST (assayed in the presence of 0.24 M aspartate and 6 mM 2-oxoglutarate) was inhibited 23% by 1 mM and 59% by 5 mM perfluorosuccinate. In extracts of digitonin-treated mitochondria from rat liver, 1 mM perfluorosuccinate inhibited aspartate and alanine (assayed in the presence of 0.49 M alanine and 10 mM 2-oxoglutarate) aminotransferases by 71% and 25%, respectively, without having any effect on malate dehydrogenase activity. PEPCK activity (assayed in the presence of 16 mM phosphoenolpyruvate and 1.2 mM GDP) in extracts of hepatocytes treated with digitonin, was decreased 40–50% by 1 mM perfluorosuccinate. These data support the suggestion that inhibition of PEPCK and AST accounts for the effects of perfluorosuccinate on gluconeogenesis in hepatocytes.

Perfluorosuccinate inhibition is confined to the cytoplasm

In hepatocytes from fasted rats, approximately 97% of cellular PEPCK activity is cytoplasmic, but only 18% of AST activity is located in this compartment [25–27]. The presence of AST in both mitochondrial and cytoplasmic compartments of the cell prompts the question—is the inhibitory effect of perfluorosuccinate confined to the cytoplasm or can perfluorosuccinate penetrate the mitochondrial membrane and interact also with the mitochondrial AST? This was investigated indirectly by a study of the comparative effects of perfluorosuccinate on ureogenesis and gluconeogenesis. These pathways occur partly in both mitochondrial and cytoplasmic compartments and the mitochondrial AST has a crucial role in producing aspartate for translocation to the cytoplasm to participate in both processes [21]. Both perfluorosuccinate and quinolinate had a greater inhibitory effect on gluconeogenesis than on ureogenesis (Table 3). On the other hand, aminooxyacetate, an aminotransferase inhibitor [28, 29] which inhibits both cytoplasmic and mitochondrial aminotransferase activities [19, 21, 30, 31], decreased the rates of both gluconeogenesis and ureogenesis by at least 84% (Table 3). On the basis of these data we concluded that under the conditions used, the inhibitory effects of perfluorosuccinate are confined to the cytoplasm.

Perfluorosuccinate inhibits gluconeogenesis by inhibition of the activities of cytoplasmic PEPCK and AST

The results obtained with the above gluconeogenic precursors suggested that the inhibitory effects of perfluorosuccinate can be explained in terms of inhibition of the activities of the two cytoplasmic enzymes PEPCK and AST. The very similar inhibitory effects of perfluorosuccinate and quinolinate on gluconeogenesis from pyruvate (Table 1) are explained by inhibition of PEPCK activity alone since there is no requirement for a malate–aspartate shuttle [18–21]. On the other hand, when gluconeogenesis occurs from lactate, inhibition by perfluorosuccinate of both AST and PEPCK activities is involved, and it would appear that these effects are additive with regard to inhibition of glucose synthesis. The data in Table 4 suggest that perfluorosuccinate is not readily released by hepatocytes although fractionation studies did not reveal any irreversible inhibition of PEPCK or AST activities.

Perfluorosuccinate inhibition of the activity of cytoplasmic AST causes inhibition of ethanol oxidation

The oxidation of ethanol by hepatocytes from fasted rats is limited by the rate of re-oxidation of cytosolic NADH generated by the alcohol dehydrogenase reaction [22, 32, 33]. Consequently, it is to be expected that ethanol oxidation should be sensitive to inhibition by perfluorosuccinate of cytoplasmic AST, which is a component of the malate–aspartate shuttle. A 25% inhibition of ethanol oxidation by 1 mM perfluorosuccinate (Table 2) compares with a 23% inhibition of rat-liver cytoplasmic AST activity *in vitro*. In the presence of quinolinate, reducing equivalents from the reactions catalysed by alcohol and lactate dehydrogenases are able to be transported into the mitochondria via the malate–aspartate shuttle as normal. However, since the mitochondrial aldehyde dehydrogenase reaction which oxidises almost all of the acetaldehyde produced in the cytoplasm [33–35], is also generating reducing

Table 4. The inhibitory effects of perfluorosuccinate on gluconeogenesis and ethanol oxidation in hepatocytes, are not removed by washing the cells

Substrates	Treatment	% Inhibition of glucose rate	% Inhibition of rate of ethanol removal
2 mM palmitate, 10 mM lactate	A	61	—
1 mM pyruvate	B	74	—
8 mM ethanol, 10 mM lactate	A	91	32
	B	90	42

Hepatocytes were pre-incubated at 37° for 15 min with 0 or 1 mM perfluorosuccinate in the presence of 10 mM lactate and 1 mM pyruvate, them washed twice with cell suspension medium by low speed centrifugation. The cells were then added to mixtures containing the indicated substrates, and incubated at 37° for 35 min (Treatment A). Alternatively, cells pre-incubated in the absence of inhibitor were then incubated with the same substrates, but with 1 mM perfluorosuccinate present for the 35 min (Treatment B). The % inhibition of glucose formation or of ethanol removal was calculated relative to the case where inhibitor was excluded in both the 35 min incubation and the 15 min pre-incubation. The data are from a representative experiment.

equivalents in the absence of any enhanced demand for ATP, the net result is an increase in the total malate concentration and the mitochondrial redox state. These accumulations do not occur in the presence of perfluorosuccinate, although the mitochondrial redox state is more reduced in the presence of palmitate (not shown). The failure of the malate concentration to increase in the presence of perfluorosuccinate is explained by the fact that the inhibitor's action almost totally prevented the removal of lactate and thus, the formation of pyruvate, which normally serves as a precursor of oxaloacetate from which any accumulating malate will be derived (Table 2).

In conclusion, the data presented in this paper are compatible with perfluorosuccinate acting through the inhibition of cytoplasmic PEPCK and AST activities. Perfluorosuccinate is thus, a potentially useful (and commercially available) inhibitor of gluconeogenesis.

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