

Quinolate inhibition of gluconeogenesis is dependent on cytosolic oxalacetate concentration

An explanation for the differential inhibition of lactate and pyruvate gluconeogenesis

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In isolated rat hepatocytes, the phosphoenolpyruvate carboxykinase (PEPCK) inhibitor, quinolate decreased gluconeogenesis from lactate more than from pyruvate (78 vs 44%). Quinolate inhibition of PEPCK has been reported to be competitive with oxalacetate (OAA), and therefore higher cytosolic OAA concentrations could be expected to alleviate quinolate inhibition of PEPCK and hence reduce its effect on gluconeogenesis. With pyruvate as a carbon source, the cytosolic concentration of OAA was higher than with lactate (40 vs 9.7 μM). The levels of OAA were manipulated metabolically by adding asparagine (which provides more cytosolic OAA through the urea cycle) or oleate (which increases malate efflux from the mitochondria). In each of the 8 conditions studied, quinolate inhibition of gluconeogenesis was inversely related to the levels of OAA in the cytosol. Quinolate inhibition of asparagine gluconeogenesis was not due to a non-specific effect on urea synthesis.

Gluconeogenesis Rat hepatocyte Quinolate Phosphoenolpyruvate carboxykinase Oxalacetate
Asparagine

1. INTRODUCTION

Since the discovery in this laboratory [2-5] that quinolate (a metabolite of tryptophan) inhibits the gluconeogenic enzyme PEPCK, it has served as an extremely useful tool for the selective inhibition of this enzyme. The specificity of action has enabled many investigators to study the role of this important enzyme in the regulation of gluconeogenesis. One inconsistency in the proposed action of quinolate seemed to exist, however; quinolate inhibits gluconeogenesis from lactate to a

greater extent than from pyruvate [6-9]. Glucose synthesis from either lactate or pyruvate requires flux through PEPCK, and therefore if this were the only site of quinolate inhibition, one would expect equivalent effects of quinolate. These experiments indicated that quinolate might act at another site; such a proposition would obviously cloud previous interpretations of quinolate inhibition data.

The formation of phosphoenolpyruvate from pyruvate during gluconeogenesis involves the carboxylation of mitochondrial pyruvate to oxalacetate (OAA), which is shuttled to the cytosol in the form of malate or aspartate [10]. The demand for reducing equivalents at the glyceraldehyde-3-phosphate dehydrogenase step determines the route for the transport of C_4 acids into the cytosol, where it then serves as the substrate for PEPCK. During gluco-

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; OAA, oxalacetate

A portion of this work has been presented in preliminary form [1]

neogenesis from lactate, the reducing equivalents are formed in the production of pyruvate by lactate dehydrogenase, and therefore mitochondrial OAA is transported into the cytosol as aspartate. On the other hand, when pyruvate is the substrate, reducing equivalents must be supplied by the mitochondria via conversion of OAA to malate, followed by malate export to the cytosol and subsequent reoxidation of malate to OAA generating the needed NADH.

Based on these distinct pathways for lactate and pyruvate gluconeogenesis, the different degrees of quinolinate inhibition observed with these two substrates might be explained by quinolinate action at a secondary site unique to the lactate pathway. Quinolinate has been reported to inhibit purified aspartate aminotransferase [11], but this action does not account for the differential effect on gluconeogenesis as is apparent from ethanol oxidation studies performed by Crow et al. [8]. The rate of ethanol oxidation is known to be limited by the transfer of reducing equivalents into the mitochondria via the Borst cycle. The enzymes involved in this pathway (cytosolic and mitochondrial aspartate aminotransferases and malate dehydrogenases) are the same enzymes utilized to shuttle OAA out of the mitochondria during gluconeogenesis from either lactate or pyruvate. Ethanol oxidation (in the presence of lactate to generate Borst cycle intermediates) is insensitive to quinolinate. This indicates that none of the enzymes involved in the Borst cycle, and consequently none of the enzymes unique to the gluconeogenic pathways of pyruvate or lactate, are sufficiently affected by quinolinate to explain the differential actions of this inhibitor. The distinct responses of lactate and pyruvate gluconeogenesis to quinolinate therefore cannot be attributed to a site of quinolinate action specific to one of the two pathways. Here, we suggest that the inhibition of gluconeogenesis by quinolinate is dependent on the cytosolic concentration of the PEPCK substrate, OAA.

MATERIALS AND METHODS

2.1. Source of reagents

Quinolinate (pyridine-2,3-dicarboxylic acid) was obtained from Aldrich. 3-Mercaptopicolinate was a gift from Smith, Kline, and French, Inc. All other reagents were from Sigma.

2.2. Hepatocyte isolation and incubation

Male Sprague-Dawley rats (250–300 g) were fasted 24 h prior to use. Hepatocytes were prepared by a modification [12] of the method of Berry and Friend [13]. Preparation and suspension of cells were performed in the absence of added Ca^{2+} ; CaCl_2 was subsequently added to the incubation medium (final concentration, 2.4 mM) except where noted. Greater than 90% of the cells were judged viable based on the exclusion of trypan blue. Hepatocytes (40 mg wet wt) were incubated in 1 ml of Krebs-Henseleit buffer containing 1.5% defatted bovine serum albumin [14] and 2.4 mM CaCl_2 at 37°C in stoppered 20 ml scintillation vials flushed with 95:5% O_2 : CO_2 . Quinolinate, at 4.8 mM, (or saline in controls) was added 15 min prior to the addition of gluconeogenic substrates (10 μl) to facilitate uptake of the inhibitor [15]. Hepatocytes were subsequently incubated for 40 min with substrates, during which time the rates of gluconeogenesis were linear. In all cases, the endogenous rate of gluconeogenesis (in the absence of added substrates) was subtracted from the rate in the presence of substrate.

2.3. Metabolite determinations

Cellular metabolites were measured following termination of incubations with perchloric acid. Glucose was determined using the glucose oxidase method [16] and both lactate [17] and pyruvate [18] were assayed in extracts of incubated whole cells. Previous studies have shown that similar ratios of lactate to pyruvate are found in the cytosol, whole cells, or the suspending medium [19,20]. Urea was measured in deproteinized extracts using urease and glutamate dehydrogenase [21].

Cytosolic malate concentrations were determined by subtracting mitochondrial contents (separated by a modification [22] of the method of Zuendorf and Tager [23]) from total cellular malate levels. Briefly, following short incubations (approx. 15 s) in the absence or presence of digitonin, cells were centrifuged through an oil layer into perchloric acid. Butyl malonate (1 mM) was included during the brief incubation to inhibit malate transport across the mitochondrial membrane. The mitochondrial and total levels of malate were subsequently measured fluorometrically in neutralized perchloric acid extracts [24].

Cytosolic OAA was calculated assuming that malate dehydrogenase and lactate dehydrogenase catalyse near-equilibrium reactions [25,26] and 2 ml cytosolic water per g dry wt cells [27].

3. RESULTS AND DISCUSSION

In agreement with previous reports [6-9], quinolinate inhibited the rate of gluconeogenesis from lactate more than it did if pyruvate served as the carbon source (78.0 vs 42.1%, table 1). Using a 10:1 ratio of lactate to pyruvate, the degree of quinolinate inhibition was intermediate between the effects observed using either substrate alone. Since an effect of quinolinate on the enzymes specific to either of the gluconeogenic pathways for these substrates can be excluded (see above), a model was required to explain these results.

One hypothesis that has been advanced is that quinolinate inhibition of gluconeogenesis might be dependent upon the concentration of OAA in the cytosol [8]. Consistent with this notion, kinetic studies on purified PEPCK have indicated that quinolinate inhibition of PEPCK is competitive

[28] with OAA, although noncompetitive inhibition has also been observed [29]. The rate of gluconeogenesis is not necessarily dependent on cytosolic OAA levels [30], and therefore the application of the kinetic studies with the isolated enzyme is unclear. Nevertheless, we examined the possibility that quinolinate inhibition of glucose synthesis could be related of cytosolic OAA concentrations.

3-Mercaptopicolinate, a structural analog of quinolinate, inhibits PEPCK through a parallel mechanism greatly enhanced by the presence of Fe^{2+} [26,29]. Consistent with previous observations [30], 3-mercaptopicolinate was a more potent inhibitor of lactate than pyruvate gluconeogenesis (76 vs 54%). We have focussed our study on quinolinate inhibition of gluconeogenesis; however these results may have similar applications to the effects of 3-mercaptopicolinate.

Pyruvate is likely to produce high levels of cytosolic OAA because of the near equilibrium nature of both malate dehydrogenase and lactate dehydrogenase reactions. When pyruvate is the gluconeogenic substrate, some of it is converted to

Table 1

Quinolinate inhibition of gluconeogenesis from different substrates

| Substrates | Rate of gluconeogenesis ($\mu\text{mol}/\text{min}$ per g wet wt) | | % inhibition by quinolinate |
|------------------------------|---|-------------------|--------------------------------|
| | Control | + quinolinate | |
| Lactate | 0.432 \pm 0.010 | 0.095 \pm 0.007 | 78.0 |
| Lactate + oleate | 0.784 \pm 0.017 | 0.267 \pm 0.008 | 66.0 |
| Lactate + asparagine | 0.530 \pm 0.008 | 0.217 \pm 0.007 | 59.2 |
| Lactate + pyruvate (1 mM) | 0.473 \pm 0.016 | 0.232 \pm 0.008 | 50.9 |
| Pyruvate | 0.415 \pm 0.008 | 0.241 \pm 0.006 | 42.1 |
| Pyruvate + oleate | 0.804 \pm 0.012 | 0.490 \pm 0.010 | 38.8 |
| Pyruvate + asparagine | 0.493 \pm 0.011 | 0.320 \pm 0.009 | 35.1 |
| Asparagine | 0.232 \pm 0.008 | 0.167 \pm 0.005 | 28.0 |

Isolated hepatocytes from 24 h fasted rats were incubated with 4.8 mM quinolinate (or saline in controls) for 15 min prior to the addition of substrates. All incubations were in Krebs-Henseleit buffer containing 1.5% defatted bovine serum albumin and 2.4 mM Ca^{2+} as detailed in section 2. Initial concentrations of the added substrates were: 10 mM lactate; 10 mM pyruvate (unless otherwise indicated); 5 mM asparagine; and 1 mM oleate. Hepatocytes were incubated with substrates for 40 min and glucose production was measured as described in section 2. Results are expressed as the means \pm SE of duplicates from 4 different hepatocyte preparations

lactate thereby oxidizing the cytosol. This oxidized cytosol shifts the equilibrium of malate dehydrogenase towards OAA formation leading to the generation of high cytosolic OAA concentrations. The presence of high concentrations of lactate however, produces a far less oxidized cytosol, and therefore the malate dehydrogenase equilibrium no longer favors OAA production as it does with pyruvate. Consequently, pyruvate would be expected to produce higher OAA concentrations than lactate which could account for its lower sensitivity to quinolinate.

To determine the dependency of quinolinate inhibition on OAA levels, the cytosolic concentration of OAA was manipulated while the magnitude of glucose synthesis inhibition by quinolinate was assessed. The instability of OAA in solution renders direct addition of OAA to cells undesirable. Asparagine and oleate were chosen to increase the cytosolic OAA concentration. Asparagine is metabolized by cytosolic asparaginase [31] to aspartate and ammonia which can then be converted to urea and OAA via the urea cycle. Oleate would also be expected to raise OAA levels in the cytosol by providing a more reduced mitochondria

favoring malate efflux into the cytosol thereby increasing OAA production through mass-action on cytosolic malate dehydrogenase. These compounds were therefore added with pyruvate or lactate to increase cytosolic OAA levels to determine whether the inhibitory effects of quinolinate would be diminished.

Oleate or asparagine stimulated glucose synthesis from either lactate or pyruvate (table 1). In each case the addition of asparagine or oleate also diminished the degree of quinolinate inhibition. When asparagine was the only substrate for gluconeogenesis, the weakest effect of quinolinate was observed (only 28% inhibition).

The role of Ca^{2+} in quinolinate inhibition of glucose synthesis was explored by incubating hepatocytes in the absence of added Ca^{2+} . Under these conditions, identical degrees of inhibition were observed for the different substrate combinations (not shown), however the absolute rates of gluconeogenesis were lower, in agreement with a previous report [32].

The cytosolic concentrations of OAA were assessed to determine if they correlated with the theoretical predictions discussed above. OAA is

Table 2

Calculated cytosolic oxalacetate concentrations from cytosolic malate and the lactate/pyruvate ratio in response to different substrates

| Substrates | Cytosolic malate concentration (mM) | Lactate/pyruvate ratio | Calculated cytosolic OAA concentration (μM) |
|---------------------------|-------------------------------------|------------------------|--|
| Lactate | 0.309 \pm 0.011 | 8.04 \pm 0.14 | 9.7 \pm 0.41 |
| Lactate + oleate | 0.860 \pm 0.071 | 11.2 \pm 0.46 | 19.4 \pm 1.5 |
| Lactate + asparagine | 0.643 \pm 0.021 | 6.71 \pm 0.41 | 24.2 \pm 1.8 |
| Lactate + pyruvate (1 mM) | 1.27 \pm 0.092 | 10.1 \pm 0.87 | 32.0 \pm 2.8 |
| Pyruvate | 0.430 \pm 0.012 | 2.69 \pm 0.21 | 40.4 \pm 3.1 |
| Pyruvate + oleate | 0.829 \pm 0.065 | 4.40 \pm 0.33 | 47.6 \pm 3.4 |
| Pyruvate + asparagine | 0.588 \pm 0.028 | 2.59 \pm 0.22 | 57.4 \pm 4.7 |
| Asparagine | 0.529 \pm 0.038 | 2.11 \pm 0.26 | 69.8 \pm 6.6 |

Substrate concentrations are identical to those in table 1. Hepatocytes were incubated with 4.8 mM quinolinate for 15 min prior to the addition of gluconeogenic substrates. Determinations of lactate, pyruvate, and the separation and measurement of cytosolic malate are outlined in section 2. Cytosolic OAA was calculated based on near-equilibrium assumptions described in section 2. Results are expressed as the means \pm SE for duplicate determinations from 4 separate hepatocyte preparations

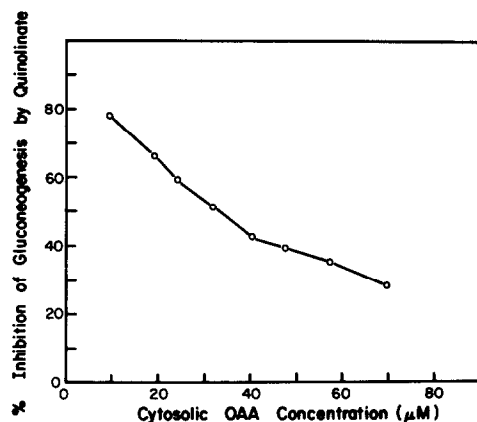


Fig.1. Correlation between cytosolic OAA concentration and quinolinate inhibition of gluconeogenesis. Data were compiled from the experiments described in tables 1 and 2.

very unstable and is present in small amounts rendering its direct measurement in cell extracts extremely difficult. An accepted alternative is to calculate the cytosolic concentration of OAA assuming near-equilibrium for the lactate and malate dehydrogenases reactions [25,26] and using measured concentrations of lactate, pyruvate and cytosolic malate (see section 2). As predicted, oleate, which increases malate efflux from the mitochondria, increased the levels of malate in the cytosol when added in combination with either lactate or pyruvate (table 2). Similarly, asparagine, which forms cytosolic fumarate through the urea cycle, elevated cytosolic malate concentrations.

More cytosolic OAA was formed during gluconeogenesis from pyruvate than lactate, in support of our hypothesis. Furthermore, both oleate and asparagine increased the cytosolic concentration of OAA measured during either lactate or pyruvate gluconeogenesis (table 2). The values for cytosolic OAA are within the range of the reported K_m for PEPCK of 11–110 μM , depending on assay conditions [33]. We also confirm previous reports that the rate of gluconeogenesis is not directly determined by the cytosolic concentration of OAA.

In tables 1 and 2, the substrates for gluconeogenesis are listed in decreasing order of quinolinate effectiveness and increasing concentrations of OAA, respectively. It is apparent that the degree of quinolinate inhibition is reduced as the cytosolic concentration of OAA increase (fig.1).

Table 3.

Effect of quinolinate on urea synthesis in isolated hepatocytes

| Substrates | Urea synthesis ($\mu\text{mol}/\text{min}$ per g wt) | |
|-----------------------|--|-----------------|
| | Control | + quinolinate |
| Pyruvate + asparagine | 1.37 \pm 0.06 | 1.33 \pm 0.08 |
| Lactate + asparagine | 1.69 \pm 0.08 | 1.72 \pm 0.08 |
| Asparagine | 1.60 \pm 0.10 | 1.64 \pm 0.11 |

Urea production was measured as described in section 2 after incubating hepatocytes with substrate concentrations indicated in table 1. All data presented are the means \pm SE of duplicates from 4 separate hepatocyte preparations

Since it was possible that quinolinate inhibition of gluconeogenesis from asparagine, where OAA is generated through the urea cycle, might be the result of a site of quinolinate action on urea synthesis, this parameter was examined. Quinolinate had no effect on the rate of urea formation from asparagine, either alone, or in the presence of pyruvate or lactate (table 3).

It is evident that the level of OAA can dictate the sensitivity of gluconeogenesis to quinolinate. The levels of OAA were manipulated through two independent mechanisms and a close inverse correlation with quinolinate inhibition was observed. It appears that the differential sensitivities of lactate and pyruvate gluconeogenesis can be accounted for by the differences in their production of OAA; no other site for quinolinate action need be invoked. In light of the similar mode of action of 3-mercaptopycolinate, it is probable that parallel conclusions may be drawn for that inhibitor.

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