Regulation of carbohydrate metabolism by 2,5-anhydro-D-mannitol

(GLUONEOGENESIS/GLYCOLYSIS/FRUCTOSE-1,6-BISPHOSPHATASE/PHOSPHOFRUCTOKINASE 1/PYRUVATE KINASE)

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ABSTRACT In hepatocytes isolated from fasted rats, 2,5-anhydro-D-mannitol inhibits gluconeogenesis from lactate plus pyruvate and from substrates that enter the gluconeogenic pathway as triose phosphate. This fructose analog has no effect, however, on gluconeogenesis from xylitol, a substrate that enters the pathway primarily as fructose 6-phosphate. The sensitivity of gluconeogenesis to 2,5-anhydro-D-mannitol depends on the substrate metabolized; concentrations of 2,5-anhydro-D-mannitol required for 50% inhibition increase in the order lactate plus pyruvate < dihydroxyacetone < glycerol < sorbitol < fructose. The inhibition by 2,5-anhydro-D-mannitol of gluconeogenesis from dihydroxyacetone is accompanied by an increase in lactate formation and by two distinct crossovers in gluconeogenic-glycolytic metabolite patterns — i.e., increases in pyruvate concentrations with decreases in phosphoenolpyruvate and increases in fructose 1,6-bisphosphate concentrations with little change in fructose 6-phosphate. In addition, 2,5-anhydro-D-mannitol blocks the ability of glucagon to stimulate gluconeogenesis and inhibit lactate production from dihydroxyacetone. 2,5-Anhydro-D-mannitol decreases cellular fructose 2,6-bisphosphate content in hepatocytes; therefore the effects of the fructose analog are not mediated by fructose 2,6-bisphosphate, a naturally occurring allosteric regulator. 2,5-Anhydro-D-mannitol also inhibits gluconeogenesis in hepatocytes isolated from fasted diabetic rats, but higher concentrations of the analog are required.

2,5-Anhydro-D-mannitol (2,5-AM-ol), an analog of β-D-fructose locked in the furan ring structure, is phosphorylated by fructokinase to form 2,5-AM-ol-1-P (1, 2).

Because 2,5-AM-ol is symmetrical, the monophosphate product can be considered an analog of both fructose-1-P and fructose-6-P (3). Because of the stability of its ring structure, 2,5-AM-ol monophosphate cannot be cleaved by aldolase in a manner similar to that of fructose-1-P, nor can it act as a substrate for phosphoglucoisomerase and be converted to glucose-6-P in a manner similar to that of fructose-6-P. 2,5-AM-ol monophosphate is a substrate for phosphofructokinase 1 (4, 5). The resulting product, 2,5-AM-ol bisphosphate, is an analog of β-fructose-1,6-P2 rather than α-fructose-1,6-P2 and, as such, is not hydrolyzed readily by fructose-1,6-bisphosphatase, which prefers the α anomer (6). The bisphosphate compound, thus, should accumulate within the cell. In vitro experiments have shown that 2,5-AM-ol bisphosphate is a competitive inhibitor of fructose-1,6-bisphosphatase (7, 8). In view of the described findings, the potential of 2,5-AM-ol to act as a regulator of gluconeogenesis and glycolysis was examined in isolated rat hepatocytes.

METHODS AND MATERIALS

Synthesis of 2,5-AM-ol. 2,5-AM-ol was prepared as in ref. 9 except the crystallization step was omitted. The crude 2,5-AM-ol, in 5 mM ammonium borate (pH 9), was purified on Dowex-1-X-8 (borate) (10). The unretained material, after deionization and repeated concentration by evaporation of methanol, showed only one spot on paper or thin-layer chromatography (5, 9). 2,5-AM-ol was stored frozen as an aqueous solution (2 M) containing 0.1 mM EDTA.

Isolation and Incubation of Hepatocytes. Unless otherwise noted, cells were isolated from livers of normal rats fasted 24 hr and were incubated as previously described (11). CaCl2 (1.3 mM) was included in all incubation mixtures. Diabetes was induced in rats fasted overnight by intravenous injection of alloxan at 40 mg/kg of body weight and was verified by blood glucose concentrations greater than 300 mg/100 ml after 1 week. Hepatocytes isolated from livers of diabetic rats fasted 26–30 hr were incubated in Krebs–Henseleit medium containing 1.3% bovine serum albumin with oxygenation at 37°C for 45 min to deplete residual glycogen stores, then washed and incubated as usual. Basal glucose production was 0.09–0.17 μmol per min per g wet weight. Similar preliminary incubation of hepatocytes isolated from fasted normal rats did not alter their behavior.

Measurement of Glucose and Lactate Production. Glucose and lactate were determined enzymatically as described (11).

Determination of Gluconeogenic-Glycolytic Intermediates. Hepatocytes (0.45–0.5 g wet weight in 12 ml total volume) were incubated under 95% O2/5% CO2 in sealed 125-m1 plastic bottles. After 15 min, two 5-ml aliquots were removed from each flask and the hepatocytes were concentrated through a bromocresol/dodecane mixture into 1.0 ml of 6% perchloric acid/1 mM isocitrate as in ref. 12. An additional 1.2 ml of each suspension was added to perchloric acid directly for determinations of total glucose and pyruvate concentrations. Duplicate samples were pooled and neutralized with KOH.

Metabolites in the neutralized extracts, except for fructose-1,6-P2, were assayed spectrophotometrically by established enzymatic methods (13). Fructose-1,6-P2 was assayed with pig kidney fructose-1,6-bisphosphatase (0.5 unit/ml final concentration) and coupled to NADPH production via phosphoglucoisomerase and glucose-6-P dehydrogenase. Pig kidney fructose-

Abbreviations: 2,5-AM-ol, 2,5-anhydro-D-mannitol; dibutyryl-cAMP, N6-O2-dibutyryladenosine 3',5'-cyclic monophosphate.
RESULTS


Effects of 2,5-AM-ol on Gluconeogenesis and Glycolysis in Hepatocytes. Increasing concentrations of 2,5-AM-ol progressively decrease the rate of glucose production from 10 mM lactate plus 1 mM pyruvate; the concentration required for 50% inhibition of the rate is 0.1–0.25 mM (Fig. 1). At concentrations greater than 2 mM, glucose synthesis from the combination substrate is almost negligible (data not shown). Although rates of gluconeogenesis are substantially lowered in the presence of 2,5-AM-ol, the ability of norepinephrine or glucagon to stimulate those rates is retained at concentrations of the analog as high as 1 mM. Higher concentrations of 2,5-AM-ol can prevent the response to the catecholamine.

The effects of 2,5-AM-ol on gluconeogenesis from substrates that enter the Embden–Meyerhof pathway as triose phosphate or fructose-6-P are compared in Fig. 2. 2,5-AM-ol inhibits gluconeogenesis from 2.5 mM dihydroxycacetone, glycerol, sorbitol, or fructose, but at concentrations as high as 2 mM (Fig. 2) or 10 mM (data not shown) it does not affect gluconeogenesis from 2.5 mM xylitol. Because xylitol is metabolized via the pentose pathway and enters the gluconeogenic pathway primarily as fructose-6-P, 2,5-AM-ol does not appear to inhibit gluconeogenesis at a site subsequent to fructose-1,6-bisphosphatase. Although gluconeogenesis from each of the substrates entering as triose phosphate is affected by the analog, higher concen-

![Graph showing effects of 2,5-anhydromannitol on glucose formation.]
either hormone on the metabolism of dihydroxyacetone are abolished by 0.1–0.25 mM 2,5-AM-ol (data not shown).

Effects of 2,5-AM-ol on Gluconeogenic–Glycolytic Intermediates. In Fig. 4, metabolite profiles in hepatocytes incubated with dihydroxyacetone and 0.5 mM 2,5-AM-ol show two distinct crossovers that could account for the observed decreases in gluconeogenesis and increases in glycolysis. The increase of fructose-1,6-P$_2$ with little change of fructose-6-P and a decrease of glucose-6-P is consistent with an inhibition of fructose-1,6-bisphosphatase, activation of phosphofructokinase, or both. Although 2,5-AM-ol bisphosphate has been reported to inhibit aldolase (24), the accumulation of fructose-1,6-P$_2$ from dihydroxyacetone indicates that such an inhibition is not significant in these experiments. The second crossover, observed as an increase in pyruvate production and a decrease in phosphoenolpyruvate accumulation, corresponds to an activation of pyruvate kinase.

Effects of 2,5-AM-ol on Hepatocyte Fructose-2,6-P$_2$ Content. Fructose-2,6-P$_2$, an inhibitor of fructose-1,6-bisphosphatase and a stimulator of phosphofructokinase, is a potent physiological regulator of gluconeogenesis and glycolysis (review, see ref. 25). Glucose, fructose, dihydroxyacetone, or xylitol elicits an increase in hepatocyte concentrations of fructose-2,6-P$_2$ (cf. refs. 26–29 and Table 1). Dibutylryl-cAMP abolishes the ability of these substrates to increase fructose-2,6-P$_2$ content above basal levels (data not shown), similar to the observed effects of glucagon in the presence of glucose (25).

Because an elevation of hepatic fructose-2,6-P$_2$ by 2,5-AM-ol could explain the observed inhibition of gluconeogenesis and stimulation of glycolysis (30–32), direct measurements of fructose-2,6-P$_2$ were performed. At concentrations as low as 0.1 mM, 2,5-AM-ol blocks most of the dihydroxyacetone-induced increase of fructose-2,6-P$_2$ concentrations and significantly reduces the increase caused by glucose or by fructose (Table 1). 2,5-AM-ol also eliminates the small increase of fructose-2,6-P$_2$ content caused by xylitol, but it does not inhibit gluconeogenesis from this substrate (Fig. 2 and Table 1). Alterations in fruc-
Table 1. Fructose-2,6-P₂ concentrations in hepatocytes from fasted rats after incubation with 0, 0.1, 0.25, and 0.5 mM 2,5-AM-ol

<table>
<thead>
<tr>
<th>Substrate</th>
<th>0 mM</th>
<th>0.1 mM</th>
<th>0.25 mM</th>
<th>0.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.04</td>
<td>0.3 ± 0.04</td>
</tr>
<tr>
<td>Glucose, 20 mM</td>
<td>14.6 ± 2.8</td>
<td>7.6 ± 0.7*</td>
<td>2.3 ± 0.2*</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>Fructose, 2.5 mM</td>
<td>10.3 ± 0.5</td>
<td>3.3 ± 0.2*</td>
<td>1.4 ± 0.1*</td>
<td>0.6 ± 0.1*</td>
</tr>
<tr>
<td>DHA, 2.5 mM</td>
<td>9.7 ± 1.1</td>
<td>0.8 ± 0.1*</td>
<td>0.4 ± 0.1*</td>
<td>0.4 ± 0.1*</td>
</tr>
<tr>
<td>Xyitol, 2.5 mM</td>
<td>2.0 ± 0.6</td>
<td>0.7 ± 0.1*</td>
<td>0.5 ± 0.1*</td>
<td>0.4 ± 0.1*</td>
</tr>
</tbody>
</table>

*P < 0.10 in comparison with no added 2,5-AM-ol.
†P < 0.05 in comparison with no added 2,5-AM-ol.

2,5-AM-ol inhibits hepatocyte gluconeogenesis from substrates that enter the pathway prior to fructose-1,6-bisphosphatase, but it does not inhibit glucose production from xylitol, a substrate that enters predominantly as fructose-6-P. This indicates that phosphoglucoisomerase and glucose-6-phosphatase are not involved in the inhibition of gluconeogenesis by 2,5-AM-ol and is consistent with a report that 2,5-AM-ol fails to inhibit glucose production from galactose (33), a substrate that is converted to glucose via glucose-1-P and glucose-6-P.

Gluconeogenesis from substrates that enter the Embden–Meyerhof pathway as triose phosphate is less sensitive to inhibition by 2,5-AM-ol than that from lactate plus pyruvate. The following order of sensitivity has been observed: dihydroxyacetone > glycerol > sorbitol > fructose (Fig. 2). Because glycerol and sorbitol require oxidation prior to conversion to glucose, utilization of these substrates is already limited by the necessity for reoxidation of excessive cytosolic NADH (34), and this may account for the decreased sensitivity of gluconeogenesis to inhibition by 2,5-AM-ol. It is also possible that the increased concentrations of α-glycerophosphate in cells metabolizing glycerol or sorbitol (11, 34) are responsible for the decreased sensitivity. Because α-glycerophosphate is an allosteric inhibitor of phosphofructokinase 1 (25), its accumulation in cells could counteract the effects of low concentrations of 2,5-AM-ol by decreasing 2,5-AM-ol-bisphosphate formation. That gluconeogenesis from fructose is least sensitive to inhibition by 2,5-AM-ol is best explained by competition between fructose and 2,5-AM-ol for phosphorylation by fructokinase and is consistent with 2,5-AM-ol metabolism occurring via fructokinase in the liver (1, 2).

The metabolite patterns (Fig. 4) indicate that the major effects of 2,5-AM-ol are a decrease in the net conversion of fructose-1,6-P2 to fructose-6-P and an increase in the net conversion of phosphoenolpyruvate to pyruvate. Because 2,5-AM-ol elicits these effects without significant depletion of cellular ATP and because cellular fructose-2,6-P2 concentrations are decreased by the compound, the monophosphate and bisphosphate derivatives of the fructose analog are probably responsible. As will be reported elsewhere, these sugar phosphates are the primary products of 2,5-AM-ol metabolism in isolated hepatocytes.

At the fructose-6-P/fructose-1,6-P2 site, 2,5-AM-ol bispahosphate may cause an inhibition of fructose-1,6-bisphosphatase in vivo analogous to that observed in vitro (7, 8) or it may mimic fructose-1,6-P2 as an allosteric activator of phosphofructokinase 1. Metabolite crossover analysis cannot distinguish between these effects, and either action could lead to the increased concentrations of fructose-1,6-P2. Under some conditions both enzymes may be inhibited, because 2,5-AM-ol monophosphate may compete with and thus inhibit the phosphorylation of fructose-6-P.

The crossover between pyruvate and phosphoenolpyruvate is consistent with a stimulation of pyruvate kinase activity. While the observed increase of fructose-1,6-P2 in cells metabolizing 2,5-AM-ol could account for this activation (35–37), direct effects of the 2,5-AM-ol bis- and monophosphates may also be important at this site. 2,5-AM-ol bisphosphate may act as an allosteric activator of pyruvate kinase, as does fructose-1,6-P2, 2,5-AM-ol monophosphate may also stimulate pyruvate kinase, since fructose-1-P is a known activator of this enzyme (38).

The decrease of fructose-2,6-P2 concentrations in the presence of 2,5-AM-ol is most likely due to the presence of 2,5-AM-ol monophosphate. Because 2,5-AM-ol lacks the 2 OH it cannot be phosphorylated in the 2 position but might act as an inhibitor
of phosphofructokinase 2 and account for the ability of 2,5-AM-ol to decrease fructose-2,6-P_2 concentrations to the basal values even when substrates are present.

Inhibition by 2,5-AM-ol of gluconeogenesis from lactate plus pyruvate is more pronounced in hepatocytes isolated from normal rats rather than in those from diabetic rats (Figs. 1 and 5). Decreases in phosphofructokinase and pyruvate kinase activities have been observed in diabetic livers (39–42); either of these changes could contribute to a diminished sensitivity to 2,5-AM-ol. The observation that gluconeogenesis from dihydroxyacetone is only slightly less sensitive to 2,5-AM-ol inhibition in the diabetic cells than in the normal controls indicates that the metabolism of the fructose analog is not seriously hindered in the diabetic hepatocytes despite the lower activity of phosphofructokinase. Therefore, the relative insensitivity of gluconeogenesis from lactate plus pyruvate to inhibition by 2,5-AM-ol probably reflects the decreased amounts of pyruvate kinase, because less reversal of gluconeogenesis could be accomplished by complete activation of this enzyme.

This paper demonstrates that 2,5-AM-ol can inhibit gluconeogenesis in isolated rat hepatocytes and implicates the key gluconeogenic and glycolytic enzymes — fructose-1,6-bisphosphatase, phosphofructokinase, and pyruvate kinase — as possible sites where the mono- and bisphosphate forms of 2,5-AM-ol may be exerting an effect. More detailed studies of the metabolism of 2,5-AM-ol in hepatocytes and the effects of the phosphorylated derivatives of 2,5-AM-ol on isolated preparations of each of these key enzymes will be reported elsewhere.

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References