The Unique Role of the Kidney in Gluconeogenesis in the Chicken

THE SIGNIFICANCE OF A CYTOSOLIC FORM OF PHOSPHOENOLPYRUVATE CARBOXYKINASE

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The role of mitochondrial phosphoenolpyruvate carboxykinase in both hepatic and renal gluconeogenesis was studied in the chicken since this species reportedly lacks the cytosolic enzyme. Lactate is the major substrate used for glucose synthesis in isolated chicken hepatocytes but pyruvate, glycerol, and some amino acids, as well as lactate, are converted to glucose in isolated chicken kidney tubules. Renal gluconeogenesis and lactate production from pyruvate can be stimulated by ethanol. Amino acids also support a net production of ammonia by kidney tubules. Gluconeogenesis from all substrates and lactate production from pyruvate were stimulated by starvation (48 h), while ammoniagenesis was increased only from alanine. Ammonium chloride acidosis (7 days) had no effect on lactate or pyruvate metabolism but did increase the production of both glucose and ammonia from amino acids. These results are explained, in part, by differences in the intracellular distribution of phosphoenolpyruvate carboxykinase in the liver and kidney of the chicken. The predominantly mitochondrial (>95%) location of the liver enzyme results in an inability to provide cytosolic reducing equivalents required for gluconeogenesis. However, a significant portion of the renal enzyme is found in the cytosol (21% in fed birds; 40–50% in starved or acidotic birds). The presence of a distinct cytosolic enzyme in kidney but not liver has been confirmed immunochemically. It is proposed that, in the chicken, the liver (mitochondrial phosphoenolpyruvate carboxykinase) functions in gluconeogenesis to recycle lactate carbon (Cori cycle), while the kidney (cytosolic phosphoenolpyruvate carboxykinase) is the major organ for net gluconeogenesis from substrates such as amino acids.

The intracellular distribution of hepatic P-enolpyruvate carboxykinase (GTP) (EC 4.1.1.32) is species specific. It is almost totally cytosolic in the rat but present in both cytosol and mitochondria in most other mammalian species (1). Birds, however, have a predominantly (>95%) mitochondrial form of the enzyme and, for this reason, have been used to study the role this enzyme plays in gluconeogenesis. In vitro studies with chicken and pigeon liver have shown many differences from mammals (2–7), perhaps the most striking of which is the inability of avian liver to use "oxidized" substrates (e.g., pyruvate) or amino acids for gluconeogenesis. The mechanism for this can be explained, at least in part, by the lack of cytosolic P-enolpyruvate carboxykinase activity indirectly resulting in an inability to provide cytosolic reducing equivalents required for glucose synthesis from these substrates (2, 8). However, chickens maintain high concentrations of blood glucose (>10 mm), even during prolonged starvation, and high rates of gluconeogenesis have been demonstrated in vivo (6) The question then arises as to the net synthesis of glucose from amino acids, which are presumably released from skeletal muscle during starvation. One possibility is that these substrates are converted to glucose in an extrahepatic tissue such as the kidney since it is known that avian kidney slices can produce glucose from amino acids, glycerol, and pyruvate (9–12). This implies marked differences between avian kidney and liver in either the provision of cytosolic reducing equivalents or the intracellular distribution of P-enolpyruvate carboxykinase. The present studies were designed to elucidate differences between the liver and kidney in gluconeogenesis in the chicken. The presence and metabolic consequences of an adaptive cytosolic form of P-enolpyruvate carboxykinase are reported.

MATERIALS AND METHODS

Chemicals—Collagenase was obtained from Worthington, dithiothreitol from Calbiochem, and \(^{14}C\)-labeled sodium bicarbonate from New England Nuclear. DEAE-Affi-Gel blue was from Bio-Rad, 5,5'-dithio-bis(2-nitrobenzoic acid), 5-acetyl-CoA, and a-ketoglutarate were products of Sigma. All other enzymes and coenzymes were from Boehringer.

Chickens—One-day-old white Leghorn chickens were maintained on a commercial diet and used at 4–5 weeks of age. For some experiments, food was withdrawn 48 or 96 h prior to use. Water was available ad libitum. Chickens were made acidotic by replacement of drinking water with ammonium chloride solution for 7 days (13). Prior to experimental use, blood was drawn by cardiac puncture for the determination of plasma total CO\(_2\) using a Nalcalon manometric microgasometer. For the preparation of kidney tubules and for enzyme activity determinations, chickens were killed by decapitation and exsanguination. Kidney and/or liver was rapidly removed and placed in ice-cold Krebs-Henseleit bicarbonate-buffered saline (14) (for kidney tubules) or ice-cold 0.25 M sucrose containing 5 mM Tris buffer, pH 7.4, and 1 mM dithiothreitol (for enzyme determinations). For the isolation of hepatocytes, chickens were anesthetized by injection of Nembutal (50 mg/kg body weight) in the wing vein.

Isolation Hepatocytes and Kidney Tubules—Hepatocytes were isolated according to the method by Berry and Friend (15) as modified for the chicken by Ochs and Harris (16). Isolated kidney tubules were prepared as described by Guder and Wieland (17) and modified by Watford et al. (18). Hepatocytes (20–30 mg dry weight/flask) and kidney tubules (10–20 mg dry weight/flask) were incubated as described previously (19) except that albumin was omitted in experiments with kidney tubules. Incubations were carried out at 38 °C and stopped at 30 min by the addition of perchloric acid. After neutralization of the acid extracts, the metabolites were measured as outlined previously (20).

Measurements of Enzyme Activities—Approximately 1 g of tissue was homogenized in 10 volumes of ice-cold 0.25 M sucrose containing 10 mM Tris-HCl, pH 7.4, with 1 mM dithiothreitol and 0.2 mM P-enolpyruvate. The homogenate was centrifuged at 300 g for 5 min to remove cellular debris and the crude supernatant was used for determination of enzyme activities. The enzyme assays were performed in a reaction mixture containing 100 mM Tris-HCl, pH 7.4, 5 mM MgCl\(_2\), 0.5 mM EDTA, 5 mM ATP, 0.5 mM GTP, 100 mM 2-oxoglutarate, 0.2 mM NADP, 0.2 mM NADPH, and 100 mM phosphate buffer (pH 7.4) for the determination of P-enolpyruvate carboxykinase (GTP), P-enolpyruvate carboxykinase (ATP), and P-enolpyruvate carboxykinase (ADP), respectively. The reactions were initiated by the addition of the substrate and the reaction rates were followed at 37 °C. The enzyme activities were determined in parallel experiments for comparison of the inhibitory effects of competitive and non-competitive inhibitors in both the cytosolic and mitochondrial fractions of chicken liver and kidney.
Renal P-enolpyruvate Carboxykinase

5 mM Tris, pH 7.4, and 1 mM dithiothreitol by four passes of a motor-driven Teflon pestle homogenizer (clearance, 0.125 mm). The homogenates were centrifuged at 100,000 × g for 20 min to yield a particulate and cytosol fraction and the pellet was then washed twice. This pellet contains nuclei and cell debris, as well as mitochondria. Preliminary studies have shown that in this pellet only the mitochondria contained significant P-enolpyruvate carboxykinase activity so that we routinely measure mitochondrial enzyme activity using this fraction. Mitochondrial enzymes were “solubilized” by freezing and thawing three times.

Cross-contamination of the fractions was estimated from measurements of marked enzymes. Lactate dehydrogenase was assayed (21) as a marker for the cytosol ( Routinely <5% of the activity was found in the mitochondrial fraction). However both citrate synthase (22) and glutamate dehydrogenase (23) were used as mitochondrial markers (we routinely found 5-8% of both activities present in the cytosolic fraction). P-enolpyruvate carboxykinase was assayed in the direction of oxaloacetate formation using the method of Ballard and Hanson (24). All enzymes were assayed at 38 °C using at least two different concentrations of enzyme and the values presented have been corrected for cross-contamination of the subcellular fractions.

Preparation of Antiserum—P-enolpyruvate carboxykinase was purified from chicken liver mitochondria essentially as described by Noce and Utter (25). Antibodies were raised by injecting about 1 mg of purified enzyme mixed with Freund’s complete adjuvant into male rabbits. Additional injections of enzyme were made over a 1-month period, after which the blood was withdrawn by heart puncture. The IgG fraction was obtained by 40% ammonium sulfate fractionation and further purified on DEAE-Affigel blue equilibrated with 20 mM Tris-Cl, pH 8.0, containing 28 mM NaCl and 0.02% sodium azide. The IgG fraction did not remain on the column was concentrated and dialyzed against phosphate-buffered saline, pH 7.4. The specificity of the antibody was checked by Ouchterlony double diffusion analysis and immuneelectrophoresis. In both cases, one continuous precipitin line was observed using the purified enzyme, mitochondrial extract, or liver cytosolic fraction with no detectable spurts (data not shown).

RESULTS

Production of Glucose and Lactate in Liver and Kidney of Fed and Fasted Chickens—Previous studies (3, 7) which show that lactate is the preferred gluconeogenic substrate in chicken liver as confirmed in Table I, in which we demonstrate lower rates of gluconeogenesis from pyruvate, propionate, and glycerol, but no glucose synthesis from amino acids. The results obtained with kidney tubules were markedly different. Lactate was still the major substrate for gluconeogenesis but pyruvate conversion to glucose was approximately 34% of the rate noted with lactate. Also, there was an increase in the rate of gluconeogenesis after 48-h starvation. In contrast to the liver, substrates such as glycerol, propionate, and some amino acids (glutamine, glutamate, alanine, aspartate, and proline) were also good precursors for glucose synthesis in kidney tubules.

Further differences between the liver and kidney are shown by the effect of octanoate or ethanol on gluconeogenesis from lactate or pyruvate (Table I). In hepatocytes, octanoate (1 mM) had no effect on gluconeogenesis from either substrate, while ethanol (5 mM) stimulated gluconeogenesis from pyruvate by 55% with no effect on lactate. However, in kidney tubules from 48-h starved birds, octanoate inhibited gluconeogenesis from lactate but had no significant effect on pyruvate conversion to glucose. Also, in tubules from starved birds, ethanol increased the rate of gluconeogenesis from pyruvate to over 80% of the rate observed with lactate alone.

The synthesis of lactate and glucose from pyruvate is similar since they both require the generation of NADH in the cytosol, presumably by transport from the mitochondria. The results shown in Table II illustrate that kidney tubules isolated from chickens were able to provide such reducing power and that lactate production was increased by 48-h starvation. Furthermore, the provision of extra reducing equivalents from the oxidation of ethanol or octanoate also increased lactate production.

The Metabolism of Amino Acids and the Effect of Acidosis in Chicken Kidney—One of the major functions of mammalian kidney is to provide ammonia from glutamine for the titration of urinary acids. Acidosis produces increased renal ammoniagenesis which is accompanied by increased renal P-enolpyruvate carboxykinase activity and gluconeogenesis in the rat (26) but not in the dog (27). In tubules isolated from fed birds, glutamine, glutamate, alanine, aspartate, and proline were converted to glucose (Table I) and all except proline were good substrates for ammonia production (Table III). However, glycine and serine did not support net glucose or ammonia synthesis. Starvation (48 h) increased gluconeogenesis from glutamine, glutamate, alanine, aspartate, and proline (Table I) but only stimulated ammonia production from alanine (Table III). In experiments using acidic chickens (plasma total CO2, 13.80 ± 0.86 (9) versus 30.3 ± 1.17 (5) mM for control fed birds), renal gluconeogenesis from glutamine, glutamate, alanine, and proline was further stimulated over that noted with kidney tubules from starved animals (Table I) and alanine and proline were good substrates for ammonia production (Table III).
Liver gave very high rates of ammonia production (Table I). In particular, glutamine and alanine acids of interest that although proline is metabolized via glutamate production from proline was low (Table III).

Acidosis had no effect on the rates of renal gluconeogenesis from pyruvate or lactate (Table I), which were similar to the rates noted for tubules from fed chickens. However, acidosis did abolish the octanoate stimulation of gluconeogenesis from pyruvate (Table I), while octanoate inhibition of gluconeogenesis from lactate was maintained (Table I). Also, octanoate no longer stimulated lactate formation from pyruvate in tubules from acidotic birds (Table II).

The Distribution of P-enolpyruvate Carboxykinase in the Chicken—The results reported in Tables I and II of this paper suggest that the kidney of the chicken has a mechanism for the transfer of reducing equivalents from the mitochondria to the cytosol for either gluconeogenesis or lactate production, and that this mechanism can adapt during starvation. One possible explanation would be the presence of a cytosolic P-enolpyruvate carboxykinase in the kidney but not in the liver. Less than 6.5% of total P-enolpyruvate carboxykinase activity was found in the cytosolic fraction of chicken liver and there did not appear to be any significant change in activity during starvation (Table IV). In the kidney of fed chickens, 21.5% of the total activity of the enzyme was found in the cytosol and this increased to more than 40% after starvation. Mitochondrial P-enolpyruvate carboxykinase was not significantly changed by starvation, while the total activity increased by

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Fed</th>
<th>48-h Fasted</th>
<th>Acidotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.51 ± 0.25</td>
<td>1.11 ± 0.50</td>
<td>2.51 ± 0.65</td>
</tr>
<tr>
<td>Glutamine</td>
<td>7.80 ± 0.86</td>
<td>6.60 ± 0.10</td>
<td>12.62 ± 0.66</td>
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<tr>
<td>Glutamate</td>
<td>3.84 ± 0.86</td>
<td>3.69 ± 0.29</td>
<td>6.1 ± 1.10</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.67 ± 0.53</td>
<td>5.53 ± 0.09</td>
<td>10.46 ± 0.55</td>
</tr>
<tr>
<td>Aspartate</td>
<td>3.62 ± 0.38</td>
<td>3.88 ± 0.81</td>
<td>6.34 ± 0.43</td>
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<tr>
<td>Proline</td>
<td>1.67 ± 0.23</td>
<td>1.78 ± 0.49</td>
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</tr>
<tr>
<td>Glycine</td>
<td>1.68 ± 0.20</td>
<td>1.65 ± 0.17</td>
<td>3.24 ± 0.26</td>
</tr>
<tr>
<td>Serine</td>
<td>2.03 ± 0.23</td>
<td>1.16 ± 0.26</td>
<td>3.44 ± 0.39</td>
</tr>
</tbody>
</table>

Acidosis also increased cytosolic P-enolpyruvate carboxykinase activity in the kidney to almost 50% of the total (Table IV), but neither the renal nor the hepatic mitochondrial activities was changed.

Immunological Studies—In order to determine whether the two forms of P-enolpyruvate carboxykinase measured by our cell fractionation procedures had similar immunological properties, we purified the enzyme from the mitochondria of chicken liver and prepared antibodies against it in rabbits. The mitochondrial enzyme from the liver was totally inhibited by added antibody (Fig. 1). There was, however, approximately 5% of the total activity in the cytosol fraction which did not cross-react without antibody. If we assume that antibodies against the mitochondrial enzyme do not cross-react with P-enolpyruvate carboxykinase from the cytosol (this has been demonstrated for the two forms of rat liver P-enolpyruvate carboxykinase (28)), our results indicate that less than 1% of the total hepatic P-enolpyruvate carboxykinase activity is cytosolic.
Hepatic Gluconeogenesis in the Chicken—The rates of hepatic gluconeogenesis found in the present study are similar to those previously reported (4, 16, 29) and the finding that lactate is the major substrate used is consistent with other studies (3, 4, 7). The lack of significant cytosolic P-enolpyruvate carboxykinase activity in avian liver has been cited as a possible reason for the low rates of gluconeogenesis seen with "oxidized" substrates, such as pyruvate, in this tissue (8). Lactate conversion to pyruvate provides cytosolic NADH which is then available for the triosephosphate dehydrogenase reaction in gluconeogenesis so that P-enolpyruvate formed intramitochondrially can be readily converted to glucose. However, gluconeogenesis from pyruvate (and amino acids which give rise to pyruvate or citric acid cycle intermediates) requires the transfer of reducing equivalents as well as carbon from the mitochondria to the cytoplasm. In tissues possessing cytosolic P-enolpyruvate carboxykinase, this is accomplished by the transfer of malate (8) which is then converted to oxalacetate in the cytoplasm generating cytosolic NADH. In tissues lacking cytosolic P-enolpyruvate carboxykinase, the carbon skeleton of pyruvate must leave the mitochondria as P-enolpyruvate without the transfer of reducing equivalents. A reversal of the malate-aspartate shuttle to provide cytosolic NADH is not possible since mitochondrial aspartate transcarbamoylase is unidirectional (8). Octanoate, which has marked effects on gluconeogenesis in mammalian liver, had no effect on this process in hepatocytes from chickens, a finding consistent with earlier reports (7). Ethanol, the oxidation of which provides cytoplasmic NADH, has been reported to increase (2, 3, 7) gluconeogenesis from pyruvate in avian liver. In the present study, ethanol caused a 52% stimulation in gluconeogenesis. However, it is of interest to note that even when hepatic gluconeogenesis from pyruvate is stimulated by ethanol, the maximum rate observed is still only a fraction of that seen with lactate as a substrate. This could indicate that factors other than the availability of cytosolic NADH are involved in restricting hepatic gluconeogenesis in the chicken. Glucose synthesis from glycerol does not involve flux through the P-enolpyruvate carboxykinase reaction and the low rates observed in this and other studies (3) are due to a limitation in the oxidation of α-glycerophosphate in chicken liver.

Renal Gluconeogenesis and Ammoniagenesis in the Chicken—Gluconeogenesis in avian kidney was first described by Krebs and Yoshida (9), who, using slices of kidney from chickens and pigeons, found that lactate, pyruvate, fumarate, glutamate, and proline were good precursors. The rates of glucose synthesis by chicken kidney slices were significantly higher than found in other species, a difference attributed to the low carbohydrate (boiled eggs) diet used. Fructose, succinate, α-ketoglutarate, glycerol, and alanine are also good substrates for renal gluconeogenesis (10–12). In all of these studies, the rate of gluconeogenesis from pyruvate was almost equal to that from lactate. This marked difference between hepatic and renal capacities for gluconeogenesis was also observed in the present study. Furthermore, we have confirmed that a number of physiologically important substrates can give rise to gluconeogenesis in chicken kidney but not liver. The preferred order of substrates for gluconeogenesis was lactate, pyruvate, glycerol, glutamine, alanine, glutamate, aspartate, proline, and propionate. The stimulation of gluconeogenesis from pyruvate by ethanol is in good accord with the recent findings of Wittman and Kornbichler (30) who demonstrated considerable renal alcohol dehydrogenase activity in the chicken.

The stimulation by ethanol of glucose production from pyruvate accompanied by increased lactate formation could indicate that cytosolic NADH is rate limiting for renal gluconeogenesis from this substrate, especially since ethanol increased gluconeogenesis from pyruvate to rates similar to those from lactate. The stimulation by octanoate of gluconeogenesis from pyruvate and the inhibition from lactate in kidney tubules illustrate another difference between the liver and kidney in the regulation of gluconeogenesis. The pattern of substrate effects in the chicken kidney is similar to the guinea pig, a species where P-enolpyruvate carboxykinase is located in both mitochondria and cytosol (31).

As with mammalian species, gluconeogenesis from amino acids in chicken kidney tubules is accompanied by ammonia production. Similarly, glutamine metabolism by chicken kidney gave high rates of ammoniagenesis, although it appears that alanine would also be an important physiological source of ammonia in the chicken. Chronic acidosis causes a marked increase in ammoniagenesis together with increased gluconeogenesis from amino acids, a finding in keeping with the requirement for increased amino acid catabolism in the kidney in order to provide the extra ammonia required to titrate urinary acid. The capacity for ammonia production in the chicken kidney may be related to a relatively acid urine production by the excretion of uric acid (13).

The Unique Role of Renal P-enolpyruvate Carboxykinase in Birds—There is considerable information concerning the intracellular distribution of hepatic P-enolpyruvate carboxykinase (see Ref. 1) but little information on the renal enzyme (18). In those species studied to date, the intracellular distribution of the enzyme is approximately the same in tissues (liver and kidney cortex) of the same species. In chicken liver, the enzyme is predominantly mitochondrial (1) and is not responsive to dietary or hormonal stimuli, as confirmed in the present study. There are several recent reports of a significant P-enolpyruvate carboxykinase activity in the cytosol of chicken kidney (12, 32, 33) which is induced by starvation. We also note an increase in activity of cytosolic P-enolpyruvate carboxykinase in chicken kidney during either starvation or acidosis which correlates well with the increase in gluconeogenesis seen in isolated kidney tubules. The increase in the activity of the cytosolic enzyme observed in the kidney of chickens during chronic acidosis is similar to the rat (26), where increased ammoniagenesis from glutamine is accompanied by increased gluconeogenesis. The findings that renal gluconeogenesis from lactate and pyruvate were not stimulated in acidosis, where cytosolic P-enolpyruvate carboxykinase was elevated, suggest that there is regulation at some point in the pathway other than P-enolpyruvate carboxykinase. This may also be involved in the lack of response to octanoate in tubules from acidic birds mentioned above.

2 K. Ogata, M. Watford, L. J. Brady, and R. W. Hanson, manuscript in preparation.
Based on the present study, we propose that mitochondrial P-enolpyruvate carboxykinase functions only in gluconeogenesis from lactate (Cori cycle) which may be viewed as an almost continuous process. The cytosolic enzyme, on the other hand, is involved in net glucose synthesis from substrates such as amino acids and would require adaptation in activity in response to long term stimuli such as starvation or acidosis. The presence of an inducible cytosolic P-enolpyruvate carboxykinase enzyme in chicken kidney but not liver means that the kidney is the major organ for net gluconeogenesis in this species.

REFERENCES