¹³C-n.m.r. study of citrate metabolism in rabbit renal proximal-tubule cells

Arnold W. H. JANS,*‡ Chris WINKEL,† Lisa BUITENHUIS† and Johan LUGTENBURG†

*Max-Planck-Institut für Systemphysiologie, Rheinlanddamm 201, 4600 Dortmund 1, Federal Republic of Germany, and †Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands

The metabolism of a ¹³C-labelled substrate, [3-¹³C]citrate, was monitored in rabbit renal proximal-tubule cells by ¹³C n.m.r. The relative enrichments of label in glutamate, glutamine and αβ-glucose allowed a calculation of the rate of the glutamate dehydrogenase flux relative to the flux via phosphoenolpyruvate carboxykinase. A ratio of 1.2 ± 0.05 was found. Under steady-state conditions of active gluconeogenesis, the ratio of flux through pyruvate kinase to the gluconeogenic rate was 0.97 ± 0.03 .

INTRODUCTION

The application of ¹³C-n.m.r. spectroscopy with the use of ¹³C-labelled substrates to cells and tissue not only has become a method to trace out metabolic pathways, but is also an important tool for the determination of metabolic fluxes. Cohen [1–3] determined the ratio of pyruvate carboxylase activity relative to pyruvate dehydrogenase and the ratio of the flux through pyruvate kinase relative to the gluconeogenic flux in perfused rat liver by using [3-¹³C]alanine and [2-¹³C]pyruvate.

Similar studies have also been carried out on [2-¹³C]acetate fluxes into the tricarboxylic acid cycle of perfused hearts [4].

In a recent ¹³C-n.m.r. study [5] we discussed the metabolism of [2-¹³C]glycerol and [1,3-¹³C]glycerol in rabbit renal cells of proximal convoluted tubules and determined the relative fluxes into the tricarboxylic acid cycle. In the present study we describe the use of a new ¹³C-labelled substrate, [3-¹³C]citrate, for the examination of metabolic fluxes in rabbit renal cells of proximal convoluted tubules.

The purpose of the present study is to examine whether [3-¹³C]citrate is a suitable substrate for determination of the ratios of the relative effluxes from the tricarboxylic acid cycle from the ratios of relative ¹³C enrichments in metabolic products such as glutamate, glucose and alanine, because the administration of [3-¹³C]citrate to a suspension of rabbit renal proximal convoluted tubules directly leads to a labelling of the tricarboxylic acid cycle intermediates.

MATERIALS AND METHODS

Solutions

Standard bicarbonate-containing Ringer's solution had the following composition: NaCl, 128 mM; KCl, 5 mM; CaCl₂, 1 mM; MgSO₄, 1.2 mM; NaHCO₃, 5 mM; lactate, 0.4 mM; alanine, 0.2 mM; adenosine, 0.3 mM. pH was adjusted to 7.4 with CO₂/air (1:19). For cell wash procedures, we used a Ringer's solution containing 5 mM-glucose.

Isolation of rabbit renal proximal-tubule cells

This was carried out according to the method of Balaban *et al.* [6]. After isolation, cells were incubated with 3.5 mM-[3-¹³C]citrate.

Incubations

After isolation, cells were washed twice with bicarbonate-containing Ringer's solution and resuspended in a solution containing 3.5 mM-[3-¹³C]citrate. The incubation was performed in glass tubes in a water bath at 37 °C. During this procedure, the cell suspension was aerated with CO₂/O₂ (1:19). After 30 min, the cell pellet was spun down and used for preparation of the HClO₄ extract. The extract was freeze-dried and dissolved in 0.5 ml of ²H₂O, and the pH was adjusted to 7.4 with 0.1 M-NaO²H and 0.1 M-²HCl.

All incubations were performed at least three times.

N.m.r. spectroscopy

All ¹³C-n.m.r. spectra were recorded in 10 mm tubes on a Bruker AM 400 WB spectrometer, with the use of a broad-band probehead that was tuned to 100.61 MHz. Acquisition parameters were as follows: sweep width, 23 kHz; pulse width, 12.0 μs (corresponding to a 90° flip angle); data size, 16 k; relaxation delay, 1.7 s. Proton decoupling was done by a standard composition pulse sequence (Waltz). All n.m.r. measurements were performed at 5 °C.

The chemical shifts (δ p.p.m.) were referred to the chemical shift of lactate (C3), which was 20.95 p.p.m. [7] under our conditions.

The calculation of relative enrichments was done by correcting the integrated line intensities for nuclear Overhauser effects and relaxation times by using standard solutions, containing 0.1 M-glutamate, 0.1 M-glutamine and 0.1 M-glucose or 0.1 M-alanine. The ratio of the relative enrichments C^x_i/C^x_j of the C_i and C_j carbon atoms in glutamate, glutamine, glucose or alanine can be calculated from the equation:

$$C^x_i/C^x_j = (C_i^{\text{exp.}}/C_j^{\text{exp.}})/(C_i^{\text{mod.}}/C_j^{\text{mod.}})$$

‡ To whom correspondence should be addressed.

where $C_i^{\text{exp.}}$ and $C_j^{\text{exp.}}$ are the line intensities of the carbon atoms C_i and C_j in the ^{13}C -n.m.r. spectrum of HClO_4 extracts and $C_i^{\text{mod.}}$ and $C_j^{\text{mod.}}$ are in the corresponding integrated line intensities obtained from ^{13}C -n.m.r. spectra of model solutions, which were recorded and transformed under similar conditions to those for the spectra of the HClO_4 extracts.

Chemicals

[3- ^{13}C]Citrate was prepared from [2- ^{13}C]acetic acid as described in ref. [8]. All other chemicals were purchased from Sigma Chemie G.m.b.H., Deisenhofen, West Germany.

RESULTS AND DISCUSSION

Incubation of renal proximal-tubule cells with [3- ^{13}C]citrate

Fig. 1 shows the ^{13}C -n.m.r. spectrum of an HClO_4 extract of rabbit renal proximal-tubule cells incubated with 3.5 mM-[3- ^{13}C]citrate for 30 min at 37 °C. This spectrum shows the following interesting features. First, the major resonances can be unambiguously assigned to the C3 and C2 carbon atoms of glutamate and glutamine, whereas a resonance at 75.3 p.p.m. can be assigned to the C3 carbon atom of non-utilized citrate. Secondly, less-intense resonances are due to the carbon atoms of $\alpha\beta$ -glucose, which is labelled on C1, C2, C4, C5 and C6. Thirdly, minor resonances correspond to alanine (C2 and C3), lactate (C2 and C3), serine (C3), glutamate (C4) and glutamine (C4).

The scrambling of the ^{13}C label originating from [3- ^{13}C]citrate can be explained by a simplified schematic representation (Scheme 1), which is derived from the model representation of the tricarboxylic acid cycle by Chance *et al.* [9].

This scheme indicates that [3- ^{13}C]citrate (C3) is converted in different steps into C3-labelled 2-oxoglutarate (K3), which in turn gives rise via the glutamate dehydrogenase reaction to C3-labelled glutamate. Glutamate (C3) in turn is partly converted into C3-labelled glutamine by glutamine synthase. The fraction

Scheme 1. Simplified representation of the fate of the label from [3- ^{13}C]citrate in the tricarboxylic acid cycle, in accordance with Chance *et al.* [9]

Abbreviations: C, citrate; K, 2-oxoglutarate; S, succinate; M, malate; O, oxaloacetate; ϕPpck , flux through phosphoenolpyruvate carboxykinase; ϕGl , flux through glutamate dehydrogenase.

of 2-oxoglutarate labelled at C3 (K3) that is scrambled through the tricarboxylic acid cycle is converted into C2-labelled succinate, which gives rise to both C2- and C3-labelled malate because of the equivalent carbon atoms (C2 and C3) in succinate and fumarate.

So far, C2- and C3-labelled malate (M2 and M3) are transformed into identically labelled oxaloacetate (O2 and O3 respectively), which in turn gives rise to C3- and C2-labelled citrate (C3 and C2) by the citrate synthase reaction.

Thus 50 % of 2-oxoglutarate (K3) that is scrambled through the tricarboxylic acid cycle is transformed into C2-labelled 2-oxoglutarate (K2) after one complete turn of the cycle. The fate of intermediate C2-labelled 2-oxoglutarate (K2) can be seen in Scheme 1.

The ^{13}C label of intermediate 2-oxoglutarate (C2) that does not leave the tricarboxylic acid cycle as C2-labelled

Fig. 1. 100.6 MHz ^{13}C -n.m.r. spectrum of an HClO_4 extract of renal proximal-tubule cells incubated with 3.5 mM-[3- ^{13}C]citrate

Table 1. Ratios of relative ^{13}C enrichments, derived from the ^{13}C -n.m.r. spectrum of Fig. 1, that represent effluxes from the tricarboxylic acid cycle

Abbreviations: G_i represents the sum of the relative enrichment of the isotopomers i of glutamate and glutamine (represents the glutamate pool G_i); g_i represents the ratio of the relative enrichment of the glutamate isotopomer C_i relative to the C_i isotopomer of glutamine.

Ratio of relative labelling	Reflects
$\frac{\alpha\beta\text{-Glc5,2}}{\alpha\beta\text{-Glc6,1}} = 2.29 \pm 0.05$	Ratio of relative enrichment in oxaloacetate (C2) relative to labelling of oxaloacetate (C3)
$\frac{G3}{G2} = 2.78 \pm 0.05 = \phi\text{Gl}$	Flux through glutamate dehydrogenase
$g2 = 1.05 \pm 0.03$	Flux through glutamine synthase
$g3 = 1.04 \pm 0.03$	Flux through glutamine synthase
$\text{Ala2}/\text{Ala3} = 2.23 \pm 0.02$	Pyruvate kinase activity

glutamate disappears from the tricarboxylic acid cycle as CO_2 . The upfield region of the ^{13}C -n.m.r. spectrum of Fig. 1 shows only minor resonances due to glutamate (C1), glutamine (C1) and HCO_3^- , indicating that no full cycling of the ^{13}C label occurred under our experimental conditions.

The resonances of enriched $\alpha\beta$ -glucose and labelled alanine, lactate, glutamate (C4) and glutamine (C4) represent an efflux of ^{13}C label from the tricarboxylic acid cycle via phosphopyruvate carboxykinase.

Effluxes from the tricarboxylic acid cycle in renal proximal-tubule cells

As has been discussed above, the schematic representation in Scheme 1 shows that the relative enrichments of glucose represent the efflux of ^{13}C label from the tricarboxylic acid cycle through phosphoenolpyruvate carboxykinase and indirectly reflect the flux through gluconeogenic enzymes. The relative enrichments of glutamate and glutamine, however, represent the flux of ^{13}C label from 2-oxoglutarate into the glutamate pool.

The data of the ^{13}C -n.m.r. spectrum of Fig. 1 allow the determination of the ratios of the relative enrichments in metabolic intermediates, such as $\alpha\beta$ -glucose, the glutamate pools ($G2$ and $G3$) and alanine. These values are given in Table 1.

Since the relative enrichments of $\alpha\beta$ -glucose (C6,1 and C5,2) reflect the intermediate labelling of oxaloacetate (C2 and C3) (see Scheme 2), whereas the C2- and C3-labelled glutamate and glutamine reflect the flux through glutamate dehydrogenase (Scheme 1), we can estimate the ratio of the flux through glutamate dehydrogenase (ϕGl) relative to the flux through phosphoenolpyruvate carboxykinase (ϕPpck) by the following equation:

$$\frac{\phi\text{Gl}}{\phi\text{Ppck}} = \frac{G3/G2}{\alpha\beta\text{-Glc5,2}/\alpha\beta\text{-Glc6,1}} = 1.2 \pm 0.05 \quad (n = 3) \quad (1)$$

As the labelling of the C2 and C3 carbon atoms of oxaloacetate is reflected in the labelling pattern of

Scheme 2. Correlation between the intermediate labelling of oxaloacetate, phosphoenolpyruvate and glucose

From this correlation and the representation of Scheme 1, eqn. (1), which expresses the ratio of the flux through glutamate dehydrogenase and the flux through phosphoenolpyruvate carboxykinase, can also be determined. ^{13}C enrichments are indicated by *. P represents a phosphate group.

phosphoenolpyruvate (not visible in Fig. 1) and this in turn in the labelling of alanine (Scheme 3), we can estimate the flux through pyruvate kinase (ϕ_{Pk}) to the flux through glutamate dehydrogenase (ϕ_{Gl}) by eqn. (2):

$$\frac{\phi_{Pk}}{\phi_{Gl}} = \frac{Ala2/Ala3}{G3/G2} = 1.1 \pm 0.05 \quad (n = 3) \quad (2)$$

Since the relative enrichments in glucose are also a measure for the flux through phosphoenolpyruvate carboxykinase, we can determine in a similar way to that indicated above the following ratio:

$$\frac{\phi_{Pk}}{\phi_{Ppck}} = \frac{Ala2/Ala3}{\alpha\beta-Glc5,2/\alpha\beta-Glc6,1} = 0.97 \pm 0.03 \quad (n = 3) \quad (3)$$

The latter value is identical with the results obtained from incubations of renal proximal-tubule cells with $[2-^{13}C]$ glycerol and is in the same range as determined by ^{13}C n.m.r. in perfused rat livers [2].

In addition, the ratio of the relative enrichment of the glutamate *Ci* carbon atom to the relative enrichment of the glutamine *Ci* carbon atom shows constant values for C2 and C3, indicating a constant flux through glutamine synthase. So far we had determined relative ratios of effluxes from the tricarboxylic acid cycle in rabbit renal proximal-tubule cells incubated with 3.5 mM- $[3-^{13}C]$ -citrate for 30 min. To examine those effluxes that are representative for the steady-state situation *in vivo*, we determined the relative enrichments of the glutamate pool, glucose and alanine from a time course experiment (Table 2).

In Table 2 $G3/G2$ represents the labelling of the glutamate pool through glutamate dehydrogenase and $\alpha\beta-Glc5,2/\alpha\beta-Glc6,1$ represents the gluconeogenic flux. Because of the poor signal-to-noise ratio in the time-course experiment we were not able to determine the $Ala2/Ala3$ ratio from time courses.

Scheme 3. Correlation between intermediate labelling of oxaloacetate and the observed labelling of alanine, which allows with the representation of Scheme 2 the derivation of eqn. (2)

Table 2. Typical time-dependence of the relative enrichment of the glutamate pool and glucose after administration of 3.5 mM- $[3-^{13}C]$ citrate to a suspension of renal proximal-tubule cells

For details see the text. Abbreviations are as in Table 1.

<i>t</i> (min)	$\frac{G3}{G2}$	$\frac{\alpha\beta-Glc5,2}{\alpha\beta-Glc6,1}$
5	4.5	—
10	2.81	2.34
15	2.83	2.30
20	2.79	2.28
30	2.78	2.29
45	2.78	2.29
60	2.57	2.24

The data in Table 2 indicate that 15 min after administration of $[3-^{13}C]$ citrate to a suspension of rabbit renal proximal-tubule cells the different effluxes from the tricarboxylic acid cycle are in a 'steady state' and that the flux ratios from eqns. (1)–(3) are representative for the situation *in vivo*.

CONCLUSION

The data derived from the incubation of renal proximal-tubule cells with $[3-^{13}C]$ citrate show that this ^{13}C -labelled substrate is an interesting tool for the study of renal metabolism as well as for the determination of effluxes from the tricarboxylic acid cycle.

As $[3-^{13}C]$ citrate causes a direct labelling of the tricarboxylic acid-cycle intermediates and metabolic products that are a consequence of the labelling of the tricarboxylic acid cycle, the observed labelled metabolic products in the ^{13}C -n.m.r. spectrum (Scheme 3) are consistent with theoretical fate of the label of $[3-^{13}C]$ -citrate during its scrambling through the tricarboxylic acid cycle (Schemes 1–3).

So far, the ratios of the relative enrichments obtained from the ^{13}C -n.m.r. spectrum of the $HClO_4$ extract enable us to determine the ratios of label efflux from the tricarboxylic acid cycle. By using the equations derived from Schemes 1–3, we calculated efflux ratios in a steady-state labelling situation (see Table 2) in which a label efflux occurs only at the stage of 2-oxoglutarate and oxaloacetate, while common substrates such as alanine, glucose and glycerol [1–5] give rise also to an influx into the tricarboxylic acid cycle through pyruvate carboxylase and pyruvate dehydrogenase, which complicates efflux determinations. The use of other ^{13}C -labelled intermediates of the tricarboxylic acid cycle, together with the application of mathematical models [4,9] to these systems, does, however, require further investigation.

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