Enzyme-to-enzyme channelling of Krebs cycle metabolic intermediates in Caco-2 cells exposed to [2-¹³C]propionate

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The generation of ¹³C-labelled lactate by colon carcinoma cells of the Caco-2 line incubated for 120 min in the presence of [2-¹³C]propionate (10 mM) was assessed by ¹³C NMR. About 10 % of the total amount of ¹³C-labelled lactate was recovered in the cell pellet and displayed a [2-¹³C]lactate/[3-¹³C]lactate isotopomer ratio of 1.18 ± 0.01. An even higher isotopomer ratio of

1.53 ± 0.14 was observed in the case of ¹³C-labelled lactate released by the cells into the incubation medium. These findings indicate that, in the Caco-2 cells, metabolic intermediates of the Krebs cycle undergo enzyme-to-enzyme channelling in the sequence of reactions catalysed by succinyl-CoA synthetase, succinate dehydrogenase and fumarase.

INTRODUCTION

Short-chain fatty acids, such as acetate, propionate and butyrate, that are generated by colonic bacterial degradation of unabsorbed starch and non-starch polysaccharides, are metabolized in colonocytes and account for the major fraction of energy consumption in these epithelial cells [1]. Colon carcinoma cells of the Caco-2 line may, within limits, serve as an *in vitro* model for the study of colonocyte metabolism and function [2]. In the present study, Caco-2 cells were incubated in the presence of [2-¹³C]propionate and the generation of both [2-¹³C]lactate and [3-¹³C]lactate was measured by ¹³C NMR. The results provide evidence for the enzyme-to-enzyme channelling of metabolic intermediates in the Krebs (tricarboxylic acid) cycle.

MATERIALS AND METHODS

[2-¹³C]Propionate (sodium salt) was purchased from Cambridge Isotopic Laboratories (Woburn, MA, U.S.A.).

The human colon carcinoma cell line Caco-2 [3] was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). The Caco-2 cells were cultured under conditions described elsewhere [4].

Groups of about 10⁸ Caco-2 cells were each incubated for 120 min at 37 °C in 10 ml of a bicarbonate-buffered medium [5] containing BSA (0.1 mg/ml) and [2-¹³C]propionate (10 mM). Throughout incubation, the medium was equilibrated against an O₂/CO₂ mixture (95:5, v/v). After incubation and centrifugation, the incubation medium was removed and stored at -20 °C. The cell pellet was extracted with perchloric acid (5 %, v/v). The cell extract was then neutralized with KOH and treated with ethanol for glycogen precipitation [6].

The incubation media and cell extracts were eventually lyophilized, and later reconstituted in 0.5 ml of a solution of sodium formate (0.4 M) and NaN₃ (77 mM) prepared in a mixture of 85 % (v/v) H₂O and 15 % (v/v) ²H₂O.

The ¹³C spectra were recorded in the gated ¹H decoupled mode (WALTZ) on a Bruker AC250 spectrometer tuned at 62.9 MHz for ¹³C nuclei. The spectral width was 16129 Hz, the number of data points in the time domain 32 K (acquisition time = 1.016 s), the pulse length 35 °, and the number of scans 6000, with a total recycling delay of 4 s. Resonance integrated areas were determined with the standard Bruker software.

The C1/C2 and C3/C2 ratio of integrated areas in the samples were compared with those determined from a model solution of sodium lactate in natural ¹³C abundance, the spectrum of which was recorded under identical NMR acquisition and pH conditions. The isotopomer ratios of ¹³C-labelled lactate were calculated by dividing the C1/C2 or C3/C2 ratio of integrated areas in the sample by the corresponding values found in the model solution of lactate [7]. In this way, the differences between C1, C2 and C3 resonance areas due to nuclear Overhauser effects (NOE) and T₁-relaxation effects were duly accounted for.

The results are presented as mean values (± S.E.M.) together with the number of individual determinations (*n*). The statistical significance of differences between mean values was assessed by use of Student's *t*-test.

RESULTS

Naturally ¹³C-enriched lactate yielded, under the present recording conditions, C1/C2 and C3/C2 ratios of integrated areas of 0.80 and 1.65 respectively (Table 1). Control experiments indicated that propionate failed to exert any significant effect upon the C3/C2 ratio, with an overall mean value of 1.67 ± 0.12 (*n* = 12), as derived from two distinct readings of a series of six lactate samples prepared in either the absence (*n* = 3) or presence (*n* = 3) of propionate (200 mM).

In the cell extracts, obtained after 120 min incubation in the presence of [2-¹³C]propionate, the C3/C2 ratio of resonance areas for ¹³C-labelled lactate averaged 1.40 ± 0.01 (*n* = 3), a

Table 1 Resonance areas relative to that of [2-¹³C]lactate

Abbreviation: N.D., not detected. Values in parentheses indicate numbers of experiments.

Samples	[1- ¹³ C]Lactate	[2- ¹³ C]Lactate	[3- ¹³ C]Lactate
Naturally ¹³ C-enriched lactate	0.80	1.00	1.65
Cell extracts	N.D. (3)	1.00 ± 0.00 (3)	1.40 ± 0.01 (3)
Incubation medium	0.28 ± 0.03 (3)	1.00 ± 0.00 (3)	1.09 ± 0.09 (3)

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Figure 1 Representative NMR spectrum obtained in the reconstituted incubation medium of Caco-2 cells exposed to [2-¹³C]propionate

The abbreviations propC₁, propC₂ and propC₃ refer to the C1, C2 and C3 of propionate, and lactC₁, lactC₂ and lactC₃ to the C1, C2 and C3 of lactate.

value significantly lower ($P < 0.005$) than that found with naturally ¹³C-enriched lactate. From these data, it was calculated that the ¹³C abundance of propionate-derived lactate yielded a C2/C3 isotopomer ratio of 1.18 ± 0.01 . An even more obvious preferential labelling of the C2 was observed in the case of lactate released by the Caco-2 cells into the incubation medium (Table 1 and Figure 1). Thus, the C3/C2 ratio in resonance areas averaged 1.09 ± 0.09 ($P < 0.03$, as compared with 1.65), yielding a C2/C3 isotopomer ratio of 1.53 ± 0.14 . The C3/C2 ratio in resonance areas in the incubation medium averaged $77.5 \pm 7.4\%$ of the paired value found in the cell extract.

A small amount of [1-¹³C]lactate was also found in the incubation medium (Table 1). It yielded a C1/C2 isotopomer ratio averaging 0.35 ± 0.03 ($n = 3$). No sizeable amount of [1-¹³C]lactate was found in the cell extract. It should be emphasized, however, that the signal-to-noise ratio was much lower in the cell extracts than in the samples prepared from the incubation medium. The intracellular pools of [2-¹³C]lactate and [3-¹³C]lactate indeed only represented $10.5 \pm 1.7\%$ ($n = 6$) of the paired total amount of the same isotopomer recovered in both the cell extract and incubation medium. After 120 min incubation, the overall production of [2-¹³C]lactate and [3-¹³C]lactate averaged $0.97 \pm 0.16 \mu\text{mol}$ ($n = 3$).

DISCUSSION

Under the present experimental conditions, the bulk of [2-¹³C]propionate present in the incubation medium at the onset of incubation was recovered in this medium after 120 min incubation. The generation of ¹³C-labelled lactate indeed accounted for no more than about 1% of the total amount of [2-¹³C]propionate available for metabolism by the Caco-2 cells. The amount of ¹³C-labelled lactate produced by these cells was

nevertheless sufficient to achieve reliable ¹³C resonance area determinations.

The stepwise conversion of [2-¹³C]propionate into [2-¹³C]propionyl-CoA, [2-¹³C]methylmalonyl-CoA and [3-¹³C]succinyl-CoA eventually leads to the generation of succinate labelled on one of its methylene carbons. If this symmetrical intermediate did not conserve a specific molecular orientation when transferred from succinyl-CoA synthase to succinate dehydrogenase, equal amounts of [2-¹³C]lactate and [3-¹³C]lactate would eventually be generated through the sequence of reactions catalysed by succinate dehydrogenase, fumarase, NADP-malate dehydrogenase and lactate dehydrogenase. The present findings indicate, however, that more [2-¹³C]lactate than [3-¹³C]lactate is generated by Caco-2 cells exposed to [2-¹³C]propionate. This implies a conserved molecular orientation of succinate during its transfer from succinyl-CoA synthetase to succinate dehydrogenase and of fumarate during its transfer from the latter enzyme to fumarase. If fully efficient, such oriented transfers would result in the sole generation of [2-¹³C]lactate.

The finding that the preferential labelling of the C2, relative to C3, of lactate was more marked in the case of extracellular than intracellular lactate could conceivably reflect the time-related recirculation of ¹³C-labelled metabolites in the Krebs cycle, that would eventually result in the randomized distribution of ¹³C atoms. Such a process of recirculation is supported by the finding that the Caco-2 cells released a sizeable amount of [1-¹³C]lactate. It would indeed account for a lower C2/C3 isotopomer ratio in the lactate found inside the Caco-2 cells after 120 min incubation than in the extracellular lactate, which was released throughout incubation. In other words, the disparity between intracellular and extracellular lactate, in terms of the C2/C3 isotopomer ratio, would reflect the difference between an instantaneous and cumulative measurement.

The present findings further lead to the following three proposals.

First, the fact that enzyme-to-enzyme channelling of Krebs cycle intermediates in the sequence of reaction catalysed by succinyl-CoA synthetase, succinate dehydrogenase and fumarase, previously documented in yeast cells [8], rat liver [9] and calf hepatocytes [7], is also operative in human colon carcinoma cells suggests that this process represents a universal phenomenon in all cell types and all species. It was recently argued that, in *Saccharomyces cerevisiae* exposed to [3-³H]propionate, the unequal labelling of the C2 and C3 of lactate or alanine reflects propionate metabolism by the 2-methylcitrate pathway [10]. This objection does not seem applicable, however, to mammalian cells [11].

Secondly, this process of enzyme-to-enzyme channelling should now be taken into account in the interpretation of radioactive data, such as the generation of ¹⁴CO₂ by cells exposed to nutrients labelled with ¹⁴C on distinct C atoms. For instance, the postulated channelling process will delay the generation of ¹⁴CO₂ from C2-labelled acetyl residues over successive turns in the Krebs cycle [7].

Lastly, and most importantly, the kinetics of those enzymes participating in the sequence of reactions mentioned above may

well differ when such enzymes are integrated into a single metabolon, rather than acting independently of one another.

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