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 isotope ratio of 1.18±0.01. An even higher isotope 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-toenzyme 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 106 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 NaHCO3 (77 mM) prepared in a mixture of 85% (v/v) H2O and 15% (v/v) D2O.

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 isotope 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 T2-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

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<td>Naturally 13C-enriched lactate</td>
<td>0.80</td>
<td>1.00</td>
<td>1.65</td>
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<tr>
<td>Cell extracts</td>
<td>N.D. (3)</td>
<td>1.00±0.00 (3)</td>
<td>1.40±0.01 (3)</td>
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<td>Incubation medium</td>
<td>0.28±0.03 (3)</td>
<td>1.00±0.00 (3)</td>
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‡ To whom correspondence should be addressed.
value significantly lower ($P < 0.005$) than that found with naturally $^{13}$C-enriched lactate. From these data, it was calculated that the $^{13}$C abundance of propionate-derived lactate yielded a C2/C3 isotopomer ratio of $1.18 \pm 0.01$. 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 $1.09 \pm 0.09$ ($P < 0.03$, as compared with 1.65), yielding a C2/C3 isotopomer ratio of $1.53 \pm 0.14$. 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-$^{13}$C]lactate was also found in the incubation medium (Table 1). It yielded a C1/C2 isotopomer ratio averaging $0.35 \pm 0.03$ ($n = 3$). No sizeable amount of [1-$^{13}$C]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-$^{13}$C]lactate and [3-$^{13}$C]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-$^{13}$C]lactate and [3-$^{13}$C]lactate averaged $0.97 \pm 0.16 \mu$mol ($n = 3$).

**DISCUSSION**

Under the present experimental conditions, the bulk of [2-$^{13}$C]propionate present in the incubation medium at the onset of incubation was recovered in this medium after 120 min incubation. The generation of $^{13}$C-labelled lactate indeed accounted for no more than about 1\% of the total amount of [2-$^{13}$C]propionate available for metabolism by the Caco-2 cells. The amount of $^{13}$C-labelled lactate produced by these cells was nevertheless sufficient to achieve reliable $^{13}$C resonance area determinations.

The stepwise conversion of [2-$^{13}$C]propionate into [2-$^{13}$C]propionyl-CoA, [2-$^{13}$C]methylmalonyl-CoA and [3-$^{13}$C]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-$^{13}$C]lactate and [3-$^{13}$C]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-$^{13}$C]lactate than [3-$^{13}$C]lactate is generated by Caco-2 cells exposed to [2-$^{13}$C]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-$^{13}$C]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 $^{13}$C-labelled metabolites in the Krebs cycle, that would eventually result in the randomized distribution of $^{13}$C atoms. Such a process of recirculation is supported by the finding that the Caco-2 cells released a sizeable amount of [1-$^{13}$C]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 \textit{Saccharomyces cerevisiae} exposed to [3-\textsuperscript{3}H]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 \textsuperscript{14}CO\textsubscript{2} by cells exposed to nutrients labelled with \textsuperscript{14}C on distinct C atoms. For instance, the postulated channelling process will delay the generation of \textsuperscript{14}CO\textsubscript{2} 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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