Quantification of lactate from various metabolic pathways and quantification issues of lactate isotopologues and isotopomers

Wei Zhang1, Cheng Guo1, Kezhi Jiang2, Minfeng Ying1 & Xun Hu1

13C-labeled glucose combined with chromatography and mass spectrometry enables us to decipher the percentage of lactate generated from various metabolic pathways. We showed that lactate derived from glycolysis, pentose phosphate pathway, Krebs cycle, and other sources accounted for 82–90%, 6.0–11%, 0.67–1.8%, and 1.5–7.9%, respectively, depending on different types of cells. When using glucose isotopomers ([1-13C], [3-13C], [4-13C], and [6-13C]glucose) or isotopologues ([1,2-13C2], and [1,2,3-13C3]glucose) for tracing, the ratio of lactate derived from glucose carbon 1, 2, 3 over 4, 5, 6 via glycolysis varied significantly, ranging from 1.6 (traced with [1,2-13C2]glucose) to 0.85 (traced with [6-13C]glucose), but the theoretical ratio should be 1. The odd results might be caused by intramolecular 13C, which may significantly affect lactate fragmentation under tandem mass spectrometry condition, leading to erroneous quantification. Indeed, the fragmentation efficiency of [U-13C]lactate, [2,3-13C]lactate, and [3-13C]lactate were 1.4, 1.5 and 1.2 folds higher than lactate, respectively, but [1-13C]lactate was similar to lactate, suggesting that carbon-13 at different positions could differentially influence lactate fragmentation. This observed phenomenon was inconsistent with the data based on theoretical calculation, according to which activation energies for all lactate isotopomers and isotopologues are nearly identical. The inconsistency suggested a need for further investigation. Our study suggests that calibration is required for quantifying metabolite isotopologues and isotopomers.

Cancer cells convert most incoming glucose to lactate, a metabolic hallmark called Warburg effect1, 2. Lactate and proton are important for cancer cells to survive through harsh conditions. We recently demonstrated that lactate and proton together switched cancer cells from Warburg effect to an economical metabolic mode with negligible or no net generation of lactate3 and with 90% ATP from oxidative phosphorylation4. Moreover, lactate and proton together prevented cancer cells from glucose deprivation-induced death5. The findings suggested that targeting intratumoral lactic acidosis might be considered as a therapeutic target. Indeed, our clinical study demonstrated a remarkable effect of bicarbonate on local control of hepatocellular carcinoma6.

Many other investigators have independently reported the significance of intratumoral lactic acidosis in tumor biology. Clinical studies showed that high level of lactate was a strong prognostic indicator of increased metastasis and poor overall survival7–13. Gillies and Gatenby group demonstrated that systematic and tumor pHcalkalization could inhibit carcinogenesis, tumor invasion and metastasis, and they also provided integrated models that can predict the safety and efficacy of buffer therapy to raise tumor pHc14–16 and related theoretical work17, 18. Furthermore, lactic acidosis exhibited multifaceted roles in skewing macrophages19, inhibiting the function of cytotoxic T cells20, altering cancer cell metabolism21, 22, inducing chromosomal instability23, and promoting tumor angiogenesis7, 24.

Hence, lactate generation is an interesting topic in cancer metabolic research. Glucose25 is the main sources of lactate generation in cancer cell metabolism. However, the percentage of lactate generated from glucose through

1Cancer Institute (a Key Laboratory For Cancer Prevention & Intervention, China National Ministry of Education), The Second Affiliated Hospital, Zhejiang University School of Medicine, 88 Jiefang Road, Hangzhou, China. 2Key Laboratory of Organosilicon Chemistry and Material Technology, Hangzhou Normal University, Hangzhou, China. Wei Zhang and Cheng Guo contributed equally to this work. Correspondence and requests for materials should be addressed to X.H. (email: huxun@zju.edu.cn)
Materials and Methods

Cell culture and sample preparation. 4T1 and Hela cells are maintained in RPMI-1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS). 2 × 10^4 4T1 (or Hela) cells are seeded into 96-well plate to allow attachment overnight in a humidified CO₂ incubator. The culture medium was then replaced with serum free RPMI 1640 with 6 mM [1,2-¹³C₂]glucose ([1-¹³C]-, [3-¹³C]-, [4-¹³C]-, [6-¹³C]-, [1,2,3-¹³C₃]- or [U-¹³C]glucose) and incubated for another 12 hours. Cell culture supernatant was collected by centrifugation and stored at −20 °C. 4 × 10⁶ K562 cells were seeded in serum free RPMI 1640 with 6 mM [1,2-¹³C₂]glucose ([1-¹³C]-, [3-¹³C]-, [4-¹³C]-, [6-¹³C]-, [1,2,3-¹³C₃]- or [U-¹³C]glucose) into 96-well cell culture plate. After 12 hours incubation in a humidified CO₂ incubator, cell culture supernatant was collected by centrifugation and stored at −20 °C.

Thymocytes were prepared from thymus of 4-week old female ICR mice purchased from SLRC laboratory animal (Shang Hai, China). Thymus glands were squeezed by syringe inner plunger and the suspension was filtered through 100 µm cell strainer. Thymocytes were incubated at a density of 2 × 10⁶ cells/ml in serum free RPMI 1640 with 6 mM [1,2-¹³C₂]glucose ([1-¹³C]-, [3-¹³C]-, [4-¹³C]-, [6-¹³C]-, [1,2,3-¹³C₃]- or [U-¹³C]glucose) for 12 hours in a humidified CO₂ incubator, and supernatant was collected by centrifugation and stored at −20 °C.

Prior to analysis by LC-MS/MS, 10 µl sample was diluted by 190 µl acetonitrile, and cleaned by centrifugation at 10000 g for 30 min at 4 °C, supernatant was collected and stored at −20 °C.

Enzymatic determination of lactate. The concentration of lactate is measured according to previously reported method with modification. Briefly, 10 µl cell culture supernatant was added into 590 µl assay solution containing 2 mM nicotinamide adenine dinucleotide (NAD), 10 U lactate dehydrogenase (LDH), in reaction buffer (200 mM glycine and 170 mM hydrazine, pH 9.2), mixed well, incubated at 37 °C for 30 min, and read at 340 nm against a water blank with a ultraviolet spectrophotometer reader.

Analysis of Lactate by LC-MS/MS. Lactate isotopomers and isotopologues of the cell culture supernatant were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC was performed on a Waters ACQUITY UPLC BEH Amide column application notebook with a minor modification as follows: injection volume is 5.0 µl, and the column was kept at 50 °C. The optimized gradient conditions were adopted from Waters ACQUITY UPLC BEH Amide column application notebook with a minor modification as follows: 0-0.4 min hold for 99.9% eluent A, 0.4–0.5 min from 99.9% – 60% eluent A, 0.5–2 min from 60% – 30% eluent A, 2–2.1 min from 30% – 99.9% eluent A, and hold for 10 min. The retention time of lactate and lactate isotopomers and isotopologues was 1.29 min.

The MS detection was performed on a 4000 QTRAP mass spectrometer (AB SCIEX, Foster City, CA, USA) equipped with an ESI ion source (TurboSpray) operated in negative ion mode. Instrument control, data acquisition, and processing were performed using the Analyst 1.5.2 software. Firstly, collision-induced dissociation (CID) experiment of lactic acid standard was performed in product ion scan mode and the spectrum was illustrated in Supplementary Fig. S1A. The dissociation mechanism was proposed as illustrated in Supplementary Fig. S1B. Then MS/MS data were acquired in the multiple reaction monitoring (MRM) mode. The transition of precursor ion and the most abundant product ion (m/z 89.0 > 43.0) was monitored for quantitative determination. The ion transition m/z 89.0 > 71.0 was monitored for qualitative analysis to confirm the identity of lactate in samples. To determine the percentage of ¹³C-labeled lactate derived from ¹³C-labeled glucose through glycolysis and pentose phosphate pathway, the following ion transitions were monitored to determine the distribution of ¹³C-labeled lactate: m/z 90.0 > 43.0, [1-¹³C]lactate; m/z 90.0 > 44.0, [2-¹³C]- and [3-¹³C]lactate; m/z 91.0 > 44.0, [1,2-¹³C₂]- and [1,3-¹³C₂]lactate; m/z 91.0 > 45.0, [2,3-¹³C₃]lactate; and m/z 92.0 > 45.0, [U-¹³C]lactate.

To increase sensitivity, the ion source temperature (TEM) was set at 500 °C, and the ion spray voltage (IS) was set at −4.5 kV. Ion source gas 1 (GS1) and ion source gas 2 (GS2) used as the nebulizing and drying gases were set at 50 and 40 psi, respectively. Curtain gas (CUR) was set at 40 psi. The optimized MS conditions used for the analysis of the target analytes were shown in Supplementary Table S1.

Eliminate peak area of natural ¹³C-labeled lactate. Chemical structure of lactate and the numbering of its carbon, hydrogen and oxygen atom are shown in Supplementary Fig. S2, according to isotopic abundances of carbon, hydrogen, oxygen elements (Supplementary Table S2) and proposed fragmentation mechanism of lactate (Supplementary Fig. S1B), the relative percentage of each natural ¹³C-labeled lactate (m/z 90 > 44, 90 > 43, 91 > 45, 91 > 44 and 92 > 45) could be calculated (Supplementary Tables S3–S4). We used formula (1) to exclude the peak area of lactate isotopomers and isotopologues that from nature. Let X be the peak area of ¹³C-labeled lactate produced by cells, P_L be the measured peak area of ¹³C-labeled lactate which includes ¹³C-labeled lactate existed in nature and that produced by cells, P_U be the peak area of unlabeled lactate, R be the corresponding relative percentage of each natural ¹³C-labeled lactate.

\[ X = P_L - P_U \times R \]  

(1)

Theoretical calculations of the activation energy for fragmentation of lactate isotopomers and isotopologues. All theoretical calculations were performed by using the density functional theory (DFT).
method at the B3LYP/6-31 G(d) level of theory in the Gaussian 03 program. The optimized structures for the precursor ions, intermediates and products were identified as a true minimum in energy by the absence of imaginary frequencies. Transition states, on the other hand, were identified by the presence of one single imaginary vibration frequency with the normal vibrational mode, and further confirmed by the intrinsic reaction coordinates (IRC) analysis. The energies discussed here are the sum of electronic and thermal enthalpies. The DFT optimized structures were shown by Gauss View (version 3.07) software to give higher quality images of these structures.

Statistical analysis. All the statistical analyses were performed using SPSS statistics 19.0 software (IBM, Armonk, NY, USA). T-test was applied to evaluate the differences of peak areas ratio of different 13C-labeled lactate.

Results and Discussion

The rationale for quantification of lactate isotopomers and isotopologues. In this study, we used 7 glucose isotopomers ([1-13C]-, [3-13C]-, [4-13C]-, and [6-13C]glucose) or isotopologues ([1,2-13C2]-, [1,3-13C2]-, [2,3-13C2]-, and [U-13C]lactate) to trace lactate. Accordingly, 7 lactate isotopomers or isotopologues ([1-13C]-, [2-13C]-, [3-13C]-, [1,2-13C2]-, [1,3-13C2]-, [2,3-13C2]-, and [U-13C]lactate) would be generated.

The percentage of each lactate isotopomers or isotopologues was quantified by tandem mass spectrometry. Under MRM mode of mass spectrometer with triple quadrupole linear ion trap (QTRAP) analyzer, the precursor ion m/z 89.0 was isolated in the first quadrupole (Q1). Subsequently, the ion dissociated under collisional activation using collision gas such as nitrogen in Q2, and the fragment ions were isolated in Q3, finally, the ions were detected by the detector, as described in Supplementary Fig. S3.

As illustrated in Supplementary Fig. S1A, two fragment ions at m/z 71.0 and 43.0 were produced in the dissociation of unlabeled lactate at m/z 89.0. The transition between precursor ion and the most abundant product ion (m/z 89.0 > 43.0) was monitored for quantitative determination. And the ion transition m/z 89.0 > 71.0 was monitored for qualitative analysis to confirm the identity of lactate in samples. For 13C-labeled lactate derived from 13C-labeled glucose through glycolysis and pentose phosphate pathway, the following ion transitions were monitored to determine the distribution of 13C-labeled lactate: m/z 90.0 > 43.0, [1-13C]lactate; m/z 90.0 > 44.0, [2,1-13C]lactate and [3-13C]lactate; m/z 91.0 > 44.0, [1,2-13C2] - and [1,3-13C2]lactate; m/z 91.0 > 45.0, [2,3-13C2]lactate; and m/z 92.0 > 45.0, [U-13C]lactate.

Representative chromatograms of lactate standard and major lactate species generated in cells were shown in Supplementary Fig. 4; the sum of their percentages was more than 98% of total lactate. And the retention time of lactate and each 13C-labeled lactate generated in cells is identical to that of lactate standard. The relative standard deviation (RSD) in the peak area is about 2%, and lactate derived from mixed carbon sources (glucose and other sources) was less than 1%, which was comparable with each other. In mouse thymocytes, lactate generated from 4T1, Hela, and K562 cancer cells were comparable with each other. In mouse thymocytes, lactate generated from 4T1, Hela, and K562 cancer cells were comparable with each other. In mouse thymocytes, lactate generated from 4T1, Hela, and K562 cancer cells were comparable with each other.
2. Glucose metabolism through pentose phosphate pathway then back to glycolysis would generated 3 major species (Fig. 3), lactate ($m/z > 43$), [1,3-$^{13}C_2$]lactate ($m/z > 44$), and [3-$^{13}C$]lactate ($m/z > 44$), the ratio of ([1,3-$^{13}C_2$]lactate + [3-$^{13}C$]lactate)/lactate should be 2/3 (Fig. 3). Thus, glucose entering into pentose phosphate pathway then back to glycolysis would also generate a fraction of lactate ($m/z > 43$).

3. According to Table 1, the other sources generated about 2% of lactate ($m/z > 43$).

Thus, the percentage of lactate from glycolysis, pentose phosphate pathway, and other sources were about 90%, 6.6%, and 2.2%, respectively (the down part of Table 2), accounting for about 99% of total lactate. Using Hela and K562, we obtained nearly identical results (Table 2). For thymocytes, the percentage of lactate from glycolysis, pentose phosphate pathway, and other sources were about 85%, 6.0%, and 7.9%, respectively, accounting for about 99% of total lactate (Table 2).

Then we used [3-$^{13}C$]glucose to trace lactate generation from glycolysis and pentose phosphate pathway by 4T1 cells and calculated the percentage of lactate (Table 3). The calculation is similar as described above. In theory (Fig. 4), glycolysis would generate 2 species, [1-$^{13}C$]lactate ($m/z > 44$) derived from glucose carbons 1, 2, 3, and, lactate ($m/z > 43$) derived from glucose carbons 4, 5, 6; glucose metabolism through pentose phosphate

Table 1. The percentage of lactate isotopomers and isotopologues (mean ± SD, n = 12) generated by 4T1, Hela, K562, and thymocytes, traced by [U-$^{13}C$]glucose. 4T1, Hela, K562 and thymocytes were incubated in serum-free RPMI 1640 medium containing 6 mM [U-$^{13}C$]glucose for 12 hours in a humidified CO$_2$ incubator and culture supernatant was collected for LC-MS/MS analysis as described in Materials and Methods. Data are mean ± SD, n = 12, from 2 independent experiments.

|       | Lactate derived from [U-$^{13}C$]glucose | Lactate derived from mixed carbon sources | Lactate derived from other sources |
|-------|----------------------------------------|------------------------------------------|----------------------------------|
|       | [U-$^{13}C$]lactate                      | [2-$^{13}C$]- or [3-$^{13}C$]lactate     | [1-$^{13}C$]lactate |
|       |                                        | [1,2-$^{13}C_2$]- or [1,3-$^{13}C_2$]lactate | [2,3-$^{13}C_2$] lactate |
| 4T1   | 97% ± 0.18%                             | 0.12% ± 0.021%                           | 0.033% ± 0.0067%               |
|       |                                        | 0.32% ± 0.025%                           | 0.20% ± 0.017%                 |
|       |                                        | 2.2% ± 0.16%                             | 26 ± 2.4 (μmol/million cell/12 h) |
| Hela  | 97% ± 0.24%                             | 0.13% ± 0.050%                           | 0.34% ± 0.071%                 |
|       |                                        | 0.032% ± 0.0092%                         | 0.29% ± 0.048%                 |
|       |                                        | 2.3% ± 0.093%                            | 40 ± 3.5 (μmol/million cell/12 h) |
| K562  | 98% ± 0.21%                             | 0.11% ± 0.032%                           | 0.28% ± 0.036%                 |
|       |                                        | 0.016% ± 0.0079%                         | 0.29% ± 0.029%                 |
|       |                                        | 1.5% ± 0.12%                             | 7.9 ± 0.90 (μmol/million cell/12 h) |
| Thymocyte | 90% ± 0.17%                           | 0.23% ± 0.022%                           | 0.41% ± 0.031%                 |
|       |                                        | 0.36% ± 0.030%                           | 0.80% ± 0.041%                 |
|       |                                        | 7.9% ± 0.19%                             | 62 ± 0.83 (μmol/million cell/12 h) |

Figure 1. A time course of generation of [2,3-$^{13}C_2$]lactate (from glucose carbon 1, 2, 3) and lactate (from glucose carbon 4, 5, 6) (n = 12) from 4T1, Hela, K562 and thymocyte. Cells were incubated in serum free RPMI-1640 containing 6 mM [1,2-$^{13}C_2$]glucose and supernatant was collected for LC-MS analysis as described in Materials and Methods. Data were mean ± SD, n = 12, from 2 independent experiments.
pathway then back to glycolysis would generate 3 major species (Fig. 4), lactate (m/z 89 > 43), [1,2-13C]lactate (m/z 91 > 44), and [2-13C]lactate (m/z 90 > 44), the ratio of ([1,2-13C]lactate + [2-13C]lactate)/lactate should be 2/3. Thus, the percentage of lactate from glycolysis, pentose phosphate pathway, and other sources were about 89%, 9.2%, and 2.2%, respectively (Table 3).

Using Hela and K562, we obtained similar results (Table 3). For thymocytes, the percentage of lactate from glycolysis, pentose phosphate pathway, and other sources were about 84%, 8.1%, and 7.9%, respectively (Table 3).

Table 2. The percentage of lactate isotopomers and isotopologues (mean ± SD, n = 12) generated by 4T1, Hela, K562, and thymocytes, traced by [1,2-13C2]glucose (upper part), the percentage of lactate (mean ± SD, n = 12) derived from glycolysis, PPP and other sources (down part). 4T1, Hela, K562 and thymocyte were incubated in serum-free RPMI-1640 medium containing 6 mM [1,2-13C2]glucose for 12 hours in a humidified CO2 incubator and culture supernatant was collected for LC-MS/MS analysis as described in Materials and Methods. The upper part summarizes the percentage of all lactate isotopologues. The down part assigned the percentage of isotopologues that are generated from glycolysis, pentose phosphate pathway (calculated based on percentage of [3-13C]- and [1,3-13C2]lactate as described in corresponding text), and other sources (according to percentage listed in Table 1). Data are mean ± SD, n = 12, from 2 independent experiments.

|                | [2,3-13C2]lactate | lactate | [1,2-13C] or [3-13C]lactate | [1,2-13C] or [1,3-13C2]lactate | [1-13C]lactate | [U-13C]lactate | Total generated Lactate |
|----------------|-------------------|---------|-----------------------------|-----------------------------|----------------|------------------------|-------------------------|
| 4T1            | 56% ± 0.43%       | 40% ± 0.36% | 1.9% ± 0.099%              | 0.74% ± 0.039%              | 0.043% ± 0.032% | 1.1% ± 0.041%        | 27 ± 2.4 (μmol/million cell/12h) |
| Hela           | 55% ± 1.1%        | 41% ± 0.55% | 2.4% ± 0.25%               | 0.81% ± 0.13%              | 0.11% ± 0.10%  | 1.5% ± 0.18%         | 40 ± 1.3 (μmol/million cell/12h) |
| K562           | 56% ± 0.41%       | 40% ± 0.46% | 2.1% ± 0.069%              | 0.83% ± 0.046%              | 0.054% ± 0.032% | 1.1% ± 0.062%        | 7.7 ± 0.90 (μmol/million cell/12h) |
| Thymocytes     | 52% ± 0.48%       | 44% ± 0.40% | 1.8% ± 0.15%               | 0.59% ± 0.037%              | 0.19% ± 0.052% | 1.3% ± 0.021%        | 60 ± 1.7 (μmol/million cell/12h)  |

|                | Lactate derived from glycolysis | Lactate derived from nonoxidative PPP | Lactate derived from other sources |
|----------------|-------------------------------|--------------------------------------|-----------------------------------|
| Glc carbon 1,2,3 | [1,2-13C]lactate | [3-13C]lactate | [1,3-13C2]lactate | Glc carbon 2,3 | Glc carbon 4,5,6 | lactate | lactate | lactate | lactate |
| 4T1            | 56% ± 0.43%       | 34% ± 0.41%     | 1.9% ± 0.099%              | 0.74% ± 0.039%              | 4.0% ± 0.18%     | 2.2% ± 0.16%         | 27 ± 2.4 (μmol/million cell/12h) |
| Hela           | 55% ± 1.1%        | 33% ± 0.26%     | 2.4% ± 0.25%               | 0.81% ± 0.13%              | 4.7% ± 0.57%     | 2.3% ± 0.093%        | 40 ± 1.3 (μmol/million cell/12h) |
| K562           | 56% ± 0.41%       | 34% ± 0.64%     | 2.1% ± 0.069%              | 0.83% ± 0.046%              | 4.4% ± 0.17%     | 1.5% ± 0.12%         | 7.7 ± 0.90 (μmol/million cell/12h) |
| Thymocytes     | 52% ± 0.48%       | 33% ± 0.48%     | 1.8% ± 0.15%               | 0.59% ± 0.037%              | 3.6% ± 0.25%     | 7.9% ± 0.19%         | 60 ± 1.7 (μmol/million cell/12h)  |

|                | sum              |           | sum              | 6.6%          |           | sum              | 7.8%          | sum              | 7.3%          | sum              | 6.0%          |
|----------------|------------------|-----------|------------------|--------------|-----------|------------------|--------------|------------------|--------------|------------------|--------------|
| 4T1            | 90%              | 6.6%      | 90%              | 7.8%         | 7.3%      | 6.0%             | 7.9%         | 56%              | 8.1%         | 7.9%             | 5.9%         |
| Hela           | 55%              | 6.6%      | 55%              | 7.8%         | 7.3%      | 6.0%             | 7.9%         | 56%              | 8.1%         | 7.9%             | 5.9%         |
| K562           | 56%              | 6.6%      | 56%              | 7.8%         | 7.3%      | 6.0%             | 7.9%         | 56%              | 8.1%         | 7.9%             | 5.9%         |
The quantification problem of lactate generated from glycolysis. When \([1,2-^{13}C_2]\)glucose was used for tracing, \([2,3-^{13}C_2]\)lactate \((m/z\ 91 > 45,\ derived\ from\ glucose\ carbons\ 1,\ 2,\ 3)\) and lactate \((m/z\ 89 > 43,\ derived\ from\ glucose\ carbons\ 4,\ 5,\ 6)\) from glycolysis constituted 56% ± 0.43% and 34% ± 0.41% of total lactate, respectively, resulting in a ratio of 1.6 (Table 2). This is a surprise, as this ratio should be 1.

When using \([3-^{13}C]\)glucose for tracing, \([1-^{13}C]\)lactate \((m/z\ 90 > 43,\ derived\ from\ glucose\ carbons\ 1,\ 2,\ 3)\) and lactate \((m/z\ 89 > 43,\ derived\ from\ glucose\ carbons\ 4,\ 5,\ 6)\) from glycolysis were 45% ± 0.30% and 44% ± 0.49% of total lactate, respectively, resulting in a ratio of 1.02, very close to 1 (Table 3).

Using 4T1, Hela, k562, and mouse thymocytes, we obtained nearly identical results (Tables 2–3), indicating that the results were highly reproducible and reliable.

Tracing lactate isotopomers and isotopologues using glucose labeled with carbon-13 at different positions. We then used \([1-^{13}C]\)glucose, \([6-^{13}C]\)glucose, \([1,2,3-^{13}C_3]\)glucose, and \([4-^{13}C]\)glucose to trace lactate. The percentages of each lactate isotopologue in 4T1 were given in the upper part of Table 4. Then we estimated the percentage of lactate generated from glycolysis, pentose phosphate pathway, and other sources (the down part of Table 4).
Again, notably, the ratio of lactate derived from glucose carbon 1, 2, and 3 over that from glucose carbon 4, 5, and 6 via glycolysis varied significantly in 4T1 cells, ranging from 1.6 ([1,2,3-13C]glucose) to 0.85 ([6-13C]glucose) (the down part of Table 4). These results were reproducible using Hela, K562, and mouse thymocytes (Supplementary Tables S7–S10).

13C-labelling of lactate at different positions differentially influences its fragmentation efficiency under tandem mass spectrometry condition. In theory, glucose labeling with 13C should not alter the percentage ratio between lactate derived from glucose carbon 1, 2, 3 and lactate derived from glucose carbon 4, 5, 6, because there is no theoretical and experimental basis that enzymes responsible for glucose metabolism can distinguish 13C from 12C.

According to the methodology of mass spectrometry, isotopomers or isotopologues which differ only at 13C or 12C should not affect the ionization and fragmentation, so that the percentage of each isotopomer or isotopologue of a metabolite could be quantified. In fact, this is the principle used to measure isotopomers or isotopologues of metabolites in metabolomic studies28–31.

However, after generating so many confusing data as described above, we suspected that carbon-13 may significantly interfere with molecule fragmentation under tandem mass spectrometry condition and interfere the quantification of each lactate isotopomer and isotopologue. Standards [2,3-13C]lactate was mixed with lactate, and the [2,3-13C]lactate/lactate concentration ratios were: 9 (9:1); 4 (8:2); 2.3 (7:3); 1.5 (6:4); 1.0 (5:5); 0.67 (4:6); 0.43 (3:7); 0.25 (2:8) and 0.11 (1:9). The mixtures were analyzed by LC-MS/MS under MRM mode in losing CO, and results shown that [2,3-13C]lactate/lactate peak area ratio were 13 ± 0.45; 6.0 ± 0.29; 3.6 ± 0.077; 2.2 ± 0.042; 1.5 ± 0.070; 1.0 ± 0.018; 0.63 ± 0.011; 0.37 ± 0.0037 and 0.17 ± 0.0029, respectively (Supplementary Table S11). Overall, [2,3-13C]lactate/lactate peak area ratio was 1.5 folds higher than [2,3-13C]lactate/lactate concentration ratio (the right panel of Fig. 5A), indicating that 13C enhanced the fragmentation rate of [2,3-13C]lactate under MRM mode in losing CO.

Then we mixed [1-13C], [3-13C], or [U-13C]lactate with lactate respectively, and their concentration ratios, analysis and calculation methods were similar as described above. While [1-13C]lactate/lactate peak area ratio was comparable with their concentration ratio (the right panel of Fig. 5B), [3-13C]lactate/lactate and [U-13C]lactate/lactate peak area ratio was 1.2 (the right panel of Fig. 5C) and 1.4 (the right panel of Fig. 5D) folds higher than...
Table 3. The percentage of lactate isotopomers and isotopologues (mean ± SD, n = 12) generated by 4T1, Hela, K562, and thymocytes, traced by [3-13C]glucose (upper part), the percentage of lactate (mean ± SD, n = 12) derived from glycolysis, PPP and other sources (down part). 4T1, Hela, K562 and thymocyte were incubated in serum-free RPMI-1640 medium containing 6 mM [3-13C]glucose for 12 hours in a humidified CO2 incubator and culture supernatant was collected for LC-MS/MS analysis as described in Materials and Methods. The upper part summarizes the percentage of each lactate isotopologues. The down part assigned the percentage of isotopologues that are generated from glycolysis, pentose phosphate pathway, and other sources. Specifically, the percentage of lactate is contributed by three parts, from glycolysis, pentose phosphate pathway (calculated based on percentage of [2-13C]- and [1,2-13C2]lactate as described in corresponding text), and other sources (according to percentage listed in Table 1). Data are mean ± SD, n = 12, from 2 independent experiments.

| 13C-labeled glucose | [2-13C]lactate | [3-13C]lactate | [U-13C]lactate | [2,3-13C2]lactate | Total generated Lactate |
|---------------------|----------------|----------------|----------------|----------------|-------------------------|
| [1-13C]glucose      | 49% ± 0.45     | 49% ± 0.45     | 0.79% ± 0.030%| 0.14% ± 0.036%| 26 ± 2.4 (μmol/million cell/12 h) |
| [6-13C]glucose      | 46% ± 0.46     | 52% ± 0.50     | 0.046% ± 0.018%| 0.024% ± 0.0037%| 26 ± 1.6 (μmol/million cell/12 h) |
| [1,2,3-13C3]glucose | 57% ± 0.38     | 42% ± 0.36     | 0.30% ± 0.039%| 0.16% ± 0.018%| 26 ± 1.2 (μmol/million cell/12 h) |
| [4-13C]glucose      | 50% ± 0.34     | 48% ± 0.33     | 0.41% ± 0.054%| 1.5% ± 0.054%| 25 ± 1.1 (μmol/million cell/12 h) |

Table 4. The percentage of lactate isotopomers and isotopologues (mean ± SD, n = 12) generated by 4T1, traced by [1-13C]-, [6-13C]-, [1,2,3-13C3]-, and [4-13C]glucose (upper part), the percentage of lactate (mean ± SD, n = 12) derived from glycolysis, PPP and other sources (down part). 4T1 cells were incubated in serum-free RPMI-1640 medium containing 6 mM [1-13C]glucose or [6-13C]-, [1,2,3-13C3]-, [4-13C]glucose for 12 hours in a humidified CO2 incubator and culture supernatant was collected for LC-MS/MS analysis as described in Materials and Methods. The upper part summarizes the percentage of all lactate isotopologues. The down part assigned the percentage of isotopologues that are generated from glycolysis, pentose phosphate pathway, and other sources. Specifically, the percentage of lactate is composed of three parts, from glycolysis, pentose phosphate pathway (calculated based on percentage of [2-13C]lactate and [1,2-13C2]lactate in Table 3), and other sources (according to percentage listed in Table 1). Data are mean ± SD, n = 12, from 2 independent experiments.
**Figure 5.** 13C-labeling of lactate at different positions differentially influences its fragmentation efficiency under tandem mass spectrometry condition. (A) Left panel: standard mixture ([2,3-13C2]lactate: lactate); red line: peak area ratios; blue line: concentration ratios. The mixtures were analyzed by LC-MS/MS under MRM mode in losing CO. [2,3-13C2]lactate/lactate peak area ratio was 1.5 folds higher than [2,3-13C2]lactate/lactate concentration ratio (right panel). Original data refers to Supplementary Table 11. (B) Left panel: standard mixture ([1-13C]lactate: lactate); red line: peak area ratios; blue line: concentration ratios. The mixtures were analyzed by LC-MS/MS under MRM mode in losing CO. [1-13C]lactate/lactate peak area ratio was comparable with [1-13C]lactate/lactate concentration ratio (right panel). Original data refers to Supplementary Table 12. (C) Left panel: standard mixture ([3-13C]lactate: lactate); red line: peak area ratios; blue line: concentration ratios. The mixtures were analyzed by LC-MS/MS under MRM mode in losing CO. [3-13C]lactate/lactate peak area ratio was 1.2 folds higher than [3-13C]lactate/lactate concentration ratio (right panel). Original data refers to Supplementary Table 13. (D) Left panel: standard mixture ([U-13C]lactate: lactate); red line: peak area ratios; blue line: concentration ratios. The mixtures were analyzed by LC-MS/MS under MRM mode in losing CO. [U-13C]lactate/lactate peak area ratio was 1.4 folds higher than [U-13C]lactate/lactate concentration ratio (right panel). Original data refers to Supplementary Table 14. The data represent mean ± SD, n = 6, and are confirmed by 2 independent experiments.
their concentration ratio, respectively, indicating that \(^{13}\text{C}\)-labelling of lactate at different positions differentially influences its fragmentation efficiency under tandem mass spectrometry condition.

The lactate and \([2,3-^{13}\text{C}_2]\)lactate (or \([1-^{13}\text{C}]\), \([3-^{13}\text{C}]\), \([U-^{13}\text{C}]\)lactate) mixed standard solutions mentioned above were analyzed by LC-MS under Q1 multiple ions mode, in order to investigate whether \(^{13}\text{C}\) affects ionization process of \(^{13}\text{C}\)-labeled lactate. Data in Supplementary Tables S11–S14 point out that \(^{13}\text{C}\) slightly affect ionization of \(^{13}\text{C}\)-labeled lactate under MS condition.

| Compound          | Reactant (kJ/mol) | TS1 (kJ/mol) | Intermediate (kJ/mol) | TS2 (kJ/mol) | Product (kJ/mol) |
|-------------------|-------------------|--------------|-----------------------|--------------|------------------|
| lactate           | 0                 | 308.4        | 133.7                 | 425.6        | 296.4            |
| \([1-^{13}\text{C}]\)lactate | 0                 | 308.4        | 133.7                 | 425.8        | 296.7            |
| \([2-^{13}\text{C}]\)lactate | 0                 | 308.5        | 133.7                 | 425.7        | 296.4            |
| \([3-^{13}\text{C}]\)lactate | 0                 | 308.4        | 133.7                 | 425.6        | 296.4            |
| \([1,2-^{13}\text{C}_2]\)lactate | 0                 | 308.5        | 133.8                 | 425.9        | 296.7            |
| \([1,3-^{13}\text{C}_2]\)lactate | 0                 | 308.4        | 133.7                 | 425.9        | 296.8            |
| \([2,3-^{13}\text{C}_2]\)lactate | 0                 | 308.5        | 133.8                 | 425.7        | 296.4            |
| \([U-^{13}\text{C}]\)lactate  | 0                 | 308.5        | 133.8                 | 426.0        | 296.8            |

Table 6. Relative energies of species involved in the fragmentation reaction routes of lactate isotopomers and isotopologues.
In order to investigate $^{13}$C effect on lactate fragmentation under different MRM mode, the lactate and $\text{[2,3-}^{13}\text{C}_2\text{lactate (or [1-}^{13}\text{C}]\text{-, [3-}^{13}\text{C}]\text{-, [U-}^{13}\text{C}\text{lactate mixed standard solutions mentioned above were analyzed by MRM mode in losing H}_2\text{O. The results pointed out that }^{13}\text{C enhanced fragmentation of [2,3-}^{13}\text{C}_2\text{-, [3-}^{13}\text{C}]\text{-, or [U-}^{13}\text{C}\text{lactate as well, but the influence was less than that under MRM mode in losing CO; and the fragmentation of [1-}^{13}\text{C}\text{lactate was not affected by }^{13}\text{C under MRM mode in losing H}_2\text{O (Supplementary Tables S11–S14).}}$

The results raised a theoretical issue: $^{13}$C and $^{12}$C differs in only one number of neutron, the chemical bond was formed by sharing the outer electrons and the strength of the bond was hardly influenced by the neutron. In tandem mass spectrometry, collision gas such as nitrogen was used and the fragmentation occurs when the collision energy was high enough to break the chemical bond. Under the same collision energy, the intensity of the fragment ion from the $^{12}$C- and $^{13}$C-labeled lactate was thought to be identical. Even if there is some difference due to the mass variability, it will be very little. However, in the present study, significant difference was observed.

**Data calibration.** We used the number 1.5 ({$[2,3-^{13}\text{C}_2]\text{lactate versus lactate}$) derived from Fig. 5 to calibrate the data acquired from 4T1 incubated with {$[1,2-^{13}\text{C}]\text{glucose (Table 2). After calibration, the ratio of lactate derived from glucose carbon 1, 2, 3 over that from glucose carbon 4, 5, 6 was nearly equal (Table 5).}$

We used the number 1.2 ({$[3-^{13}\text{C}]\text{lactate versus lactate$) derived from Fig. 5 to calibrate the data of 4T1 incubated with {$[1-^{13}\text{C}]\text{- or [6-}^{13}\text{C}]\text{glucose (Table 4), the ratio of lactate derived from glucose carbon 1, 2, 3 over that from glucose carbon 4, 5, 6, was close to 1 (Table 5).$}

We used the number 1.4 ({$[1-^{13}\text{C}]\text{lactate versus lactate$) derived from Fig. 5 to calibrate the data of 4T1 incubated with {$[1,2,3-^{13}\text{C}]\text{glucose (Table 4). After calibration, the ratio of lactate derived from glucose carbon 1, 2, 3 over that from glucose carbon 4, 5, 6 was comparable with each other (Table 5).$}

Calibrations were performed on the data of Hela, K562, and thymocytes, the calibrated data indicated that the percentages of lactate derived from glucose carbon 1, 2, 3 and 4, 5, 6 were nearly equal as well (Supplementary Tables S15–S18).

**Theoretical calculation of the activation energy of lactate fragmentation.** To obtain insights into the mechanism of lactate fragmentation, we carried out theoretical calculations at the B3LYP/6-31 G(d) level of theory to quantitatively describe the energy requirements of these reactions$^{32–34}$ and a schematic potential energy surface is illustrated in Fig. 6. The energy of transition state 2 (TS2) for losing CO is higher than that of transition state 1 (TS1) for losing $\text{H}_2\text{O, suggesting that the loss of CO process is the rate determining step for the formation of ion at } m/z 43,\text{ the relative energies of lactate isotopomers and isotopologues in the fragmentation reaction routes were shown in Table 6. The energies of TS2 of all these compounds were nearly identical, suggesting that lactate isotopomers and isotopologues should have identical fragmentation behaviors in tandem mass spectrometry. The calculation was inconsistent with the experimental data and this inconsistency is worthy of further investigation.}$

**Concluding remarks.** To the best of our knowledge, there is no report regarding the significant impact of carbon-13 labeling on fragmentation of molecules under tandem MS condition. In this study, we revealed that carbon-13 labeling could significantly interfere with lactate fragmentation. This observation is inconsistent with our theoretical calculation that the activation energy of fragmentation of lactate with or without carbon-13 labeling is nearly identical. Our study also points out that potential problems may exist in the previous studies involving quantification of isotopomers and isotopologues by tandem mass spectrometry technology. In the future, the proper calibrations for quantification of isotopomers and isotopologues would be required.

**References**

1. Warburg, O. Warburg On the origin of cancer. Science. 123, 309–314 (1956).
2. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. Cell. 144, 646–74 (2011).
3. Xie, J. et al. Beyond Warburg effect—dual metabolic nature of cancer cells. Sci. Rep. 4, 4927 (2014).
4. Wu, H., Ying, M. F. & Hu, X. Lactic acidosis switches cancer cells from aerobic glycolysis back to dominant oxidative phosphorylation. Oncotarget. 7, 40621–9 (2016).
5. Wu, H. et al. Central role of lactic acidosis in cancer cell resistance to glucose deprivation-induced cell death. The Journal of pathology. 227, 189–99 (2012).
6. Chao, M. et al. A nonrandomized cohort and a randomized study of local control of large hepatocarcinoma by targeting intratumoral lactic acidosis. E-life. 5, e15691 (2016).
7. Gatenby, R. A. & Gillies, R. J. Why do cancers have high aerobic glycolysis? Nat. Rev. Cancer. 4, 891–899 (2004).
8. Brizel, D. M. et al. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. Int. J. Radiat. Oncol. Biol., Phys. 51, 349–353 (2001).
9. Walenta, S. et al. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. Cancer Res. 60, 916–921 (2000).
10. Schwickert, G., Walenta, S., Sundfor, K., Rofstad, E. K. & Mueller-Klieser, W. Correlation of high lactate levels in human cervical cancer with incidence of metastasis. Cancer Res. 55, 4757–4759 (1995).
11. Walenta, S. et al. Correlation of high lactate levels in head and neck tumors with incidence of metastasis. The American Journal of Pathology. 150, 409–415 (1997).
12. Yokota, H. et al. Lactate, choline, and creatine levels measured by \textit{vivo} 1H-MRS as prognostic parameters in patients with non-small-cell lung cancer. Journal of Magnetic Resonance Imaging. 25, 992–999 (2007).
13. Paschen, W., Djuricic, B., Mies, G., Schmidt-Kastner, R. & Linn, F. Lactate and pH in the brain: association and dissociation in different pathophysiological states. J. Neurochem. 48, 134–139 (1987).
14. Silva, A. S., Yunes, J. A., Gillies, R. I. & Gatenby, R. A. The potential role of systemic buffers in reducing intratumoral extracellular pH and acid-mediated invasion. Cancer Res. 69, 2677–2684 (2009).
15. Robey, I. F. et al. Bicarbonate increases tumor pH and inhibits spontaneous metastases. Cancer Res. 69, 2260–2268 (2009).
16. Ibrahim-Hashim, A. et al. Systemic buffers inhibit carcinogenesis in TRAMP mice. The Journal of Urology. 188, 624–631 (2012).
17. Martin, N. K. et al. Predicting the safety and efficacy of buffer therapy to raise tumour pH: an integrative modelling study. Br. J. Cancer. 106, 1280–1287 (2012).
18. Martin, N. K. et al. A mathematical model of tumour and blood pH regulation: The HCO3-/CO2 buffering system. *Math. Biosci.* **230**, 1–11 (2011).
19. Colegio, O. R. et al. Functional polarization of tumour-associated macrophages by tumour-derived lactic acid. *Nature* **513**, 559–563 (2014).
20. Haas, R. et al. Lactate Regulates Metabolic and Pro-inflammatory Circuits in Control of T Cell Migration and Effector Functions. *PLoS Biol.* **13**, e1002202 (2015).
21. Chen, J. L. et al. The genomic analysis of lactic acidosis and acidosis response in human cancers. *PLoS Genet.* **4**, e1000293 (2008).
22. Sonveaux, P. et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J. Clin. Invest.* **118**, 3930–3942 (2008).
23. Dai, C., Sun, F., Zhu, C. & Hu, X. Tumor environmental factors glucose deprivation and lactic acidosis induce mitotic chromosomal instability—an implication in aneuploid human tumors. *PLoS One* **8**, e63054 (2013).
24. Végran, F., Boidot, R., Michiels, C., Sonveaux, P. & Feron, O. Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kB/IL-8 pathway that drives tumor angiogenesis. *Cancer Res.* **71**, 2550–2560 (2011).
25. DeBarydardins, R. J. & Cheng, T. Q’s next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* **29**, 313–24 (2010).
26. Edward, P. M. & WeiI, M. H. Rapid enzymatic measurement of blood lactate and pyruvate. *Clinical Chemistry.* **13**, 314–325 (1967).
27. Frisch MJ et al. Gaussian 03; Gaussian, Inc.: Pittsburgh, PA (2003).
28. Piskounova, E. et al. Oxidative stress inhibits distant metastasis by human melanoma cells. *Nature* **527**, 186–91 (2015).
29. Fan, J. et al. Quantitative flux analysis reveals folate-dependent NADPH production. *Nature* **510**, 298–302 (2014).
30. van Heerden, J. H. et al. Lost in transition: start-up of glycolysis yields subpopulations of nongrowing cells. *Science* **343**, 1245114 (2014).
31. Môbit, J. et al. Metabolite Profiling Identifies a Key Role for Glycine in Rapid Cancer Cell Proliferation. *Science* **336**, 1040 (2012).
32. Guo, C., Yue, L., Guo, M., Jiang, K. & Pan, Y. Elimination of benzene from protonated N-benzylindoline: benzyl cation/proton transfer or direct proton transfer? *J Am Soc Mass Spectrom.* **24**(3), 381–7 (2013).
33. Wang, S. et al. Gas-Phase Fragmentation of Protonated N,2-Diphenyl-N′-(p-Toluenesulfonyl)Ethanimidamides: Tosyl Cation Transfer Versus Proton Transfer. *J. Am. Soc. Mass Spectrom.* **26**, 1428–1431 (2015).
34. Wang, S., Dong, C., Yu, L., Guo, C. & Jiang, K. Dissociation of protonated N-(3-phenyl-2H-chromen-2-ylidene)benzenesulfonamide in the gas phase: cyclization via sulfonyl cation transfer. *Rapid Commun. Mass Spectrom.* **30**, 95–100 (2016).

**Acknowledgements**

This work has been supported in part by the China National 973 project (2013CB911303), China Natural Sciences Foundation projects (81470126) and the Fundamental Research Funds for the Central Universities, National Ministry of Education, China, all to XH.

**Author Contributions**

X.H. conceived the project, designed the study, wrote the paper; W.Z. performed experiments and data processing and calibration, wrote the paper; C.G. did the LC-MS analysis, theoretical calculation, wrote the paper; K.J. did the theoretical calculation; M.Y. did the data calibration.

**Additional Information**

Supplementary information accompanies this paper at doi:10.1038/s41598-017-08277-3

**Competing Interests:** The authors declare that they have no competing interests.

**Publisher’s note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017