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Liquid chromatography – high resolution mass spectrometry analysis of fatty acid metabolism

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Abstract

We present a liquid chromatography – mass spectrometry (LC-MS) method for long-chain and very-long-chain fatty acid analysis, and its application to ¹³C-tracer studies of fatty acid metabolism. Fatty acids containing 14 to 36 carbon atoms are separated by C₈ reversed-phase chromatography using a water-methanol gradient with tributylamine as ion pairing agent, ionized by electrospray, and analyzed by a stand-alone orbitrap mass spectrometer. The median limit of detection is 5 ng/ml with a linear dynamic range of 100-fold. Ratios of unlabeled to ¹³C-labeled species are quantitated precisely and accurately (average relative standard deviation 3.2% and deviation from expectation 2.3%). In samples consisting of fatty acids saponified from cultured mammalian cells, 45 species are quantified, with average intraday relative standard deviations for independent biological replicates of 11%. The method enables quantitation of molecular ion peaks for all labeled forms of each fatty acid. Different degrees of ¹³C-labeling from glucose and glutamine correspond to fatty acid uptake from media, *de novo* synthesis, and elongation. To exemplify the utility of the method, we examined isogenic cell lines with and without activated Ras oncogene expression. Ras increases the abundance and alters the labeling patterns of saturated and monounsaturated very-long-chain fatty acids, with the observed pattern consistent with Ras leading to enhanced activity of ELOVL4 or an enzyme with similar catalytic activity. This LC-MS method and associated isotope tracer techniques should be broadly applicable to investigating fatty acid metabolism.

Keywords

elongase; exactive; fatty acids; high resolution mass spectrometry; lipids; liquid chromatography-mass spectrometry; mass isotopomer distribution analysis; tracer studies; very-long-chain fatty acids

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INTRODUCTION

Fatty acids are an essential component of all living cells. They are continuously produced, (re)processed, integrated into lipids, and degraded by a multitude of biochemical pathways. The deregulation of these pathways has been associated with pathological conditions including obesity, diabetes, viral infections, and cancer.^{1–4} A wide variety of analytical approaches have been developed that allow for the study of fatty acid metabolism. For quantitation of long-chain fatty acids (14 – 24 carbons), gas chromatography – mass spectrometry (GC-MS) is often the preferred method.^{5,6} It provides excellent separation efficiency and sensitivity. Ionization is typically by electron impact, which yields fragmentation patterns that enable accurate fatty acid identification when searched against a database.

More in depth understanding of fatty acid metabolism requires the use of stable isotope-labeled substrates that allow the determination of conversion rates (fluxes) through metabolic pathways (of which fatty acid levels indicate the resulting equilibrium outcome).^{7,8} Commonly used isotopes are ²H (e.g. in water and fatty acids) and ¹³C (e.g. in glucose, glutamine, and acetate). By feeding cultured cells, lab animals, or human subjects these labeled substrates, the fluxes through specific pathways can be gleaned from unlabeled- to labeled-compound ratios. Isotope ratio mass spectrometry (IRMS) has found widespread use in *in vivo* experiments where focus is on bulk fluxes.⁹ This technique provides little structural information as analytes are combusted into H₂ and CO₂ during analysis. An example is the study of whole body fatty acid β -oxidation by tracking ¹³CO₂ in exhaled breath from human subjects fed ¹³C-labeled fatty acids.¹⁰ An alternative is to analyze ²H₂O (for example coming from oxidation of ²H-labeled fatty acids) in biological fluids by exchanging the ²H of water with hydrogen bound to acetone, and subsequent analysis by GC-MS.^{11,12}

More specific quantitative information with respect to synthesis and turnover of individual fatty acid species has so far also been obtained by GC-MS-based analysis of fatty acid ¹³C-labeling. An important development in this regard has been the ‘mass isotopomer distribution analysis’ (MIDA) theoretical framework.¹³ This framework considers the synthesis of fatty acids as the polymerization of acetyl-CoA units. The isotopic distributions that arise are explained by the combinatorial probabilities of the sequential incorporation of unlabeled and ¹³C-labeled acetyl-CoA units, which in turn depends on the ratio of unlabeled to labeled acetyl-CoA. The MIDA framework has been successfully applied to analyze data from various GC-MS experiments where either a subset or all isotopic forms of fatty acids were measured.^{14–17} These studies primarily focused on fatty acids containing 14–18 carbons, consistent with the MIDA framework having been developed for *de novo* fatty acid synthesis. Fatty acid elongation has not been comparably well studied, in part because analysis of longer fatty acids by GC-MS with electron impact ionization is complicated by extensive fatty acid fragmentation.¹⁷ While this could be partially addressed by pairing GC-MS with chemical ionization,¹⁸ LC-MS with electrospray ionization provides a potential alternative analytical approach.

Here, we present a method for the separation and quantitation of a broad spectrum of long-chain and very-long-chain fatty acids via C₈ reversed-phase LC coupled by electrospray ionization to high-resolution MS. While sacrificing the chromatographic resolution and electron impact fragmentation patterns of GC-MS, this LC-MS approach advantageously allows effective quantitation of (1) the full repertoire of long-chain and very-long-chain fatty acid species found in mammalian cells, with the method covering fatty acids from 14 to 36 carbons; and (2) the complete isotope labeling patterns of the molecular ion of all measured fatty acids. The first ability arises from the use of liquid chromatography, which, in addition

to long-chain fatty acids, can separate very-long-chain fatty acids.^{19,20} The latter ability arises from use of electrospray ionization, which almost exclusively produces the molecular ion, coupled to a high-resolution full scan mass spectrometer. High mass resolution is valuable for differentiating species with closely related masses, e.g., a C16:1 species with two labeled ¹³C-atoms (mass 256.2313) versus an unlabeled C16:0 species (mass 256.2402), which are unambiguously separated in the orbitrap mass analyzer (m/z 28,600 compared to instrument resolving power of 100,000). While a stand-alone orbitrap mass spectrometer is used here, similar results could in principle be obtained with other high resolution mass analyzers (e.g., time-of-flight or ion cyclotron resonance).

These methodological features enable the effective probing, using ¹³C-tracers, of key metabolic events: fatty acid uptake from exogenous sources, *de novo* fatty acid synthesis, elongation, and degradation. These capabilities are exemplified here for the analysis of saponified fatty acids from cultured immortalized baby mouse kidney epithelial cells, with or without constitutive expression of an activated variant of the Ras oncogene. Activated Ras is commonly found in human cancers including pancreatic and lung cancer. Our analysis provides initial evidence that it substantially and specifically alters very-long-chain fatty acid metabolism.

EXPERIMENTAL METHODS

Chemicals, Reagents, and Media Components

HPLC-grade water (Omnisolv, EMD chemical), LC/MS-grade methanol (Optima, Fisher Chemical), and formic acid were obtained from Fisher Scientific (Pittsburgh, PA). HPLC-grade Chloroform and hexane, tributylamine, hydrochloric acid, potassium hydroxide, acetic acid, and the majority of the fatty acid standards were purchased from Sigma-Aldrich (St. Louis, MO). Additional fatty acid standards were obtained through VWR international (West Chester, PA). Polytyrosine calibration solution was obtained from Thermo Scientific (San Jose, CA). U-¹³C-glucose (99%) and U-¹³C-glutamine (99%) were from Cambridge Isotope Laboratories (Andover, MA). DMEM cell culture medium (Mediatech Cellgro) without sodium pyruvate, PBS (HyClone), and dialyzed fetal bovine serum (dFBS, HyClone) were acquired from Fisher Scientific (Pittsburgh, PA).

Fatty Acid Nomenclature

Throughout this article, the various fatty acids are represented by 'C number of carbons : number of double bonds'. For example, the symbol for palmitate is C16:0. Where this is relevant to the discussion, the position of the first double bond from the methyl terminus will be indicated by 'n-x', where n represents the number of carbon atoms, and x is the first double-bonded carbon atom counting from the methyl terminal end of the chain. For example, oleic acid is identified by C18:1n-9.

LC-MS Instrumentation and Method Settings

The LC-MS system consisted of an Accela UPLC pump (Thermo Scientific, San Jose, CA), an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), a MistraSwitch column oven and switching device (MayLab Analytical Instruments GmbH, Vienna, Austria), and an Exactive orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). All components of the system were controlled via the Xcalibur 2.1 software. The separation was performed on a Luna C₈ reversed-phase column (150 x 2.0 mm, 3 μ m particle size, 100 Å poresize, Phenomenex, Torrance, CA) using a binary gradient, with solvent A being 97:3 water/methanol with 10 mM tributylamine and 15 mM acetic acid (pH 4.5), and solvent B 100% methanol. The gradient ran linearly from 80–99% B from 0 to 20 min, remaining at 99% B from 20 to 40 min, from 99% B to 80% B to 41 min, and

remaining steady at 80% B to 50 min to re-equilibrate the column. The flow rate was 200 $\mu\text{l}/\text{min}$. Other LC parameters were autosampler temperature 4°C, injection volume 10 μl , and column temperature 25 °C. The mass spectrometer was operated in negative mode. The electrospray settings were: sheath gas flow rate 30 (arbitrary units), auxiliary gas flow rate 10 (arbitrary units), sweep gas flow rate 5 (arbitrary units), spray voltage 3 kV, capillary temperature 325 °C, capillary voltage –50 V, tube lens voltage 100 V, and skimmer voltage –25 V. The mass spec resolution was set to 100 000 resolving power at m/z 200 and the automatic gain control (ACG) was set to high dynamic range with a maximum injection time of 100 ms. The scan range was 200 – 400 m/z in the first 20 min and 300 – 575 m/z in the subsequent 30 min. When concentrated samples were injected for the purpose of specifically analyzing very-long-chain fatty acids (C26 – C34), the mass spec was set to not scan in the first 20 min, followed by a scan range of 382 – 575 m/z in the next 30 min. The instrument was mass calibrated weekly using the polytyrosine-1,3,6 standards.

Method Validation

Linearity and limit of detection (LOD) of the LC-MS system were assessed for 17 fatty acid standards (see Table 1 for list). For each standard a 5–20 mg/ml stock solution in chloroform was made that was stored at –20 °C. From these stock solutions, mixtures containing all 17 fatty acids were made in duplicate in 90:10 methanol/water containing 0.3 M potassium hydroxide, at 0, 10, 50, 100, 200, 500, 1000, 2000 ng/ml. To mimic the sample preparation protocol for saponified fatty acids, these mixtures were then heated for 1 h at 80 °C, acidified with formic acid, extracted into hexane, dried, and reconstituted in a volume of 1:1:0.3 chloroform/methanol/water such that the original concentrations of the mixtures were maintained (see sample preparation section). The linearity for each standard was evaluated using linear regression of the observed signal with respect to concentration. LOD was defined as the concentration of the standard at which the linear regression function equaled the standard's background signal plus three times its standard deviation. For the fatty acid standards that had an apparent LOD <10 ng/ml, an additional dilution series of 0, 1, 2, 5, 10 ng/ml was run for more accurate determination of the LOD. To assess inter-run, intra-day platform quantitative reproducibility, as measured by relative standard deviation (RSD), five equivalent 6 cm tissue culture plates containing parental immortalized baby mouse kidney (iBMK) cells at 80% confluence were extracted and analyzed, and the signal intensities for all observed fatty acids compared.

The accuracy and reproducibility of isotope ratio measurement was determined by comparing the experimental M^{+1}/M^0 and M^{+2}/M^0 ratios (where M^0 is the signal for the unlabeled monoisotopic compound, M^{+1} is the signal for the compound with one ^{13}C atom, and M^{+2} the signal for the compound with two ^{13}C atoms) arising from ^{13}C natural abundance with theoretical values, at 10, 50, 100, 200, 500, 1000, 2000 ng/ml. The theoretical M^0 fraction was calculated as $(1-0.011)^N$, the M^{+1} fraction as $(N)(0.011)(1-0.011)^{(N-1)}$, and the M^{+2} fraction as $(N(N-1)/2)(0.011^2)(1-0.011)^{(N-2)}$, where N is the number of carbon atoms in the fatty acid and 0.011 is the naturally occurring fraction of ^{13}C .

Mammalian Cell Culturing and Saponified Fatty Acid Sample Preparation

Immortalized baby mouse kidney (iBMK) epithelial cells were generated as described previously.²¹ Briefly, primary kidney epithelial cells from mice double deficient for Bax and Bak ($\text{Bax}^{-/-}/\text{Bak}^{-/-}$) were immortalized by E1A and dominant-negative p53 expression (Parental cells). iBMK cells expressing human oncogenic H-Ras^{V12G} were derived by electroporation with pcDNA1.H-Ras^{V12G}, followed by zeocin selection (Ras cells). The resulting cell lines were grown in Dulbecco's modified eagle media (DMEM) without pyruvate (Cellgro), supplemented with 10% dialyzed fetal bovine serum (HyClone) in an incubator containing 5% CO_2 and ambient oxygen at 37°C. For labeling experiments,

medium was prepared from DMEM without glucose or glutamine (Cellgro), with the desired isotopic form of glucose and/or glutamine added to a final concentration of 4.5 g/l glucose and 0.584 g/l glutamine.

For fatty acid metabolic studies, cells were grown in 6 cm tissue culture plates and were harvested at 80% confluence. The sample preparation procedure for each plate was as follows: first, media was aspirated and the cells were washed twice with phosphate buffered saline (PBS, at RT), followed by addition of 1 ml 50:50 methanol/water solution containing 0.05 M HCl (−20 °C). The cells were then kept on ice until they were scraped into 1.5 ml microfuge tubes (LPS, Rochester, NY) with a cell scraper. Then, 0.5 ml chloroform (−20 °C) was added to each tube, followed by vigorous vortexing for 1 min and centrifugation at 16,000 x g for 5 min. The chloroform layer was then transferred to a glass tube and the chloroform extraction step was repeated, followed by combination of the organic layers, drying under nitrogen flow, resuspension in 1 ml of 90:10 methanol/water containing 0.3 M KOH, and saponification in a 80°C water bath for 1 h. After saponification, the samples were acidified by addition of 100 µl formic acid, extracted with 1 ml hexane (2x), dried under nitrogen flow, and resuspended in a 1:1:0.3 chloroform/methanol/water solution to a 2 µl cell volume/ml solution concentration for long-chain fatty acids and 90 µl cell volume / ml solution concentration for very-long-chain fatty acids (cell volume measured with 'PCV cell counting tubes', Fisher Scientific, Pittsburgh PA). For each analysis, 10 µl of this solution was injected into the LC-MS system.

Data Analysis

Thermo Fisher mass spectrometry RAW files were converted into mzXML files using the ReAdW program.²² All mzXML files from analyses of a single experiment were then loaded into our in-house developed open-source program 'Metabolomic Analysis and Visualization Engine' (MAVEN).²³ Peak assignment was based on the constraints that the experimental m/z value be within 2.5 ppm of the theoretical [M-H][−] form of the fatty acid under evaluation, and that the measured retention time should approximate the validated retention time (± 0.5 min) for those fatty acids for which a purified standard was commercially available. Peak intensities were calculated from Extracted Ion Chromatograms (EICs) (±2.5 ppm mass range) that were smoothed by applying a Gaussian filter to the signal intensity. Data were then transported into Excel 2007 (Microsoft, Redmond, Washington) for further processing and graphing.

For studies using U-¹³C-glucose and/or U-¹³C-glutamine, signal intensities for all possible isotopic forms were calculated in an identical fashion as the unlabeled monoisotope. The isotope labeling pattern for each fatty acid was then corrected for ¹³C natural abundance using algorithms coded in MATLAB R2006a (The MathWorks, Natick, Massachusetts).²⁴ Following correction for natural abundance of ¹³C, data processing and visualization was performed with MATLAB and Excel.

RESULTS & DISCUSSION

LC-MS method development and validation

We developed a method for the analysis of fatty acids on an ultrahigh performance LC system coupled to a stand-alone orbitrap mass spectrometer. We elected to employ a C₈ reversed-phase column and a water – methanol gradient containing tributylamine as an ion-pairing agent with a 50 minutes total cycle time. The solvents were selected to match those being used for the analysis of water-soluble metabolites on the same system.²² The setup as presented here enables facile co-implementation of the fatty acid and the water-soluble metabolite methods on the same system, as it contains an automatic column switching

apparatus. The column and gradient were chosen to enable thorough fatty acid separation, so that less abundant fatty acids and their isotope labeling patterns would not be compromised by ion suppression or interference from more abundant species.¹⁹ As is shown in Figure 1A, a two-carbon increase in fatty acid chain length increased retention time by ~2.2 min, whereas introduction of a double bond decreased the retention time by ~1.5 min (Figure 1B).

Using a collection of 17 fatty acid standards with varying carbon chain lengths and degrees of unsaturation (see Table 1), we established a low ng/ml limit of detection (LOD) and a linear dynamic range (based on observed absolute signals) of at least two orders of magnitude for most of the fatty acids. The notable exceptions were C16:0 (palmitate) and C18:0 (stearate). For these fatty acids we always detect a background signal in the blank, as is also observed using other methods of analysis.¹² In attempting to mitigate this background, we found that the solvents that we used for the sample preparation and reconstitution over time became contaminated with palmitate and stearate, presumably due to their abundant presence in the environment and on plasticware. We therefore recommend closely examining the blank peaks for these fatty acids in every experiment, and to make fresh solutions approximately weekly.

When solutions were sufficiently clean and the LC-MS system not overloaded, the contribution of the background signal of palmitate and stearate was consistently less than 5% of the peak intensity coming from our samples of saponified cellular lipids (in our case samples with a 2 μ l cell volume / ml injection solvent were appropriate).

As we planned to perform isotope-tracer studies, we examined the ability of the method to accurately quantitate isotope ratios. The isotope ratio accuracy was determined by comparing the experimental M^{+1}/M^0 and M^{+2}/M^0 ratios (where M^{+1} is the ion having one ^{13}C atom from natural abundance, M^{+2} has two ^{13}C atoms, and M^0 is the unlabeled molecular ion) to the theoretical values. The theoretical M^{+1}/M^0 ratios for the standards ranged between 0.178 and 0.334, depending on the carbon chain length (see Figure 2). The average deviation from the theoretical values (accuracy) for all standards over a three order concentration range was 2.3%. The average relative standard deviation of these ratios (precision) was 3.2%. The theoretical M^{+2}/M^0 ratios for the standards ranged between 0.015 and 0.054. The average deviation from theoretical values (accuracy) was 11.6%, with an average relative standard derivation (precision) of 6.2%. While less accurate than the M^{+1}/M^0 ratios, the M^{+2}/M^0 results indicate that useful information can be obtained for labeling percentages as low as 2%. The M^{+3} natural isotope peak, which is expected to range from 0.08% to 0.6% of the M^0 peak, was too low to detect.

Labeling of cellular fatty acids

In samples of total lipids saponified from cultured baby mouse kidney epithelial cells,²¹ we detected 45 distinct fatty acids (when injected at 2 μ l cell volume / ml concentration), with a carbon chain length range of 14 to 36 carbons (for all fatty acids detected in cell extracts, see Supplementary Table S1). The average difference in retention time between successively eluting fatty acids was 0.4 min. The intraday repeatability in signal intensity for the entire procedure of sample preparation and analysis, as measured by the relative standard deviation for five biological replicates (separate tissue culture dishes with cells grown to 80% confluence) averaged 11%.

To study the kinetics of fatty acid metabolism, cells were maintained for 0, 8, and 96 hours in cell culture medium (DMEM) containing 10% dialyzed FBS and U- ^{13}C -glucose and U- ^{13}C -glutamine (all of the glucose and glutamine in the medium was labeled). The cells were then extracted for total cellular lipids and the fatty acids were saponified (Figure 3). At 0 h, the fatty acid isotope patterns showed primarily M^0 ($^{13}\text{C}_0$) and $M+1$ ($^{13}\text{C}_1$) peaks,

consistent with the natural abundance of ^{13}C , as is exemplified by palmitate (Figure 3B). By 8 h, however, labeling is evident, with the pattern consistent with assimilation of ^{13}C -acetyl units in acetyl-CoA coming from the labeled glucose and glutamine, balanced by incorporation of the unlabeled acetyl-CoA from other sources such as fatty acid degradation (Figure 3C).

By 96 h, these patterns have reached a pseudo-steady-state, consistent with the cells having undergone four rounds of doubling by this time (for palmitate see Figure 3D, for the other fatty acids see Supplementary Figure S1). Examination of Figures 3C and D reveal a significant presence of fatty acids containing an odd number of ^{13}C atoms. Consistent with known metabolic pathways, however, LC-MS analysis of extracts of water soluble metabolites revealed that the acetyl group of acetyl-CoA is either fully labeled or unlabeled following ^{13}C -glucose and ^{13}C -glutamine feeding (data not shown). This appears inconsistent with biosynthesis of fatty acids containing an odd number of atoms from the uniformly labeled nutrients; instead, the unlabeled species arise from the natural abundance of ^{13}C , and largely disappear upon correction for natural ^{13}C abundance (see e.g., Figure 4).²⁴

Overall, the ^{13}C -labeling pattern of palmitate, the main product of fatty acid synthase, exhibits a Gaussian distribution-like profile which arises from the statistical probability of incorporating unlabeled or $^{13}\text{C}_2$ -acetyl units from acetyl-CoA (Figure 3D and Supplementary Figure S1). The centroid of this distribution depends on fractional acetyl-CoA labeling, which can also be measured directly by LC-MS.²² The direct acetyl-CoA labeling measurement (of 89% acetyl-group labeling) agrees well with the palmitate labeling pattern (Figure 3D) (90% acetyl-group labeling based on MIDA analysis, i.e., a least square fit of a binomial distribution to the experimental data). In addition to this distribution of labeled species, a strong peak for the unlabeled form is also found for all fatty acids. These unlabeled species reflect residual fatty acids from before the labeling began (minor fraction) and uptake from serum in the medium (major fraction).

To obtain fatty acids with longer carbon chain-lengths and/or units of desaturation (double bonds) than palmitate, more extensive metabolic processing is required, by a set of enzymes called elongases and desaturases.²⁵ This results in more complex ^{13}C -labeling patterns, as exemplified by the C32:1 fatty acid in Figure 4. The isotope labeling pattern of this fatty acid shows a Gaussian-like labeling pattern of highly ^{13}C -labeled species that arises from de novo synthesis of a C16:0 fatty acid and subsequent elongation and desaturation. In addition, there is a distinctive and significant set of partially labeled C32:1 fatty acids having between 4 and 16-labeled carbon atoms. These arise from elongation (and additionally desaturation if a saturated fatty acid was the original substrate) of unlabeled and/or partially labeled fatty acids that have originally been taken from the medium.

For example, the M^{+16} isotope peak of C32:1 could originate from an unlabeled C16:0 or C16:1 fatty acid being taken up by the cell from the medium, followed by subsequent elongation with $^{13}\text{C}_2$ -labeled acetyl-CoA. Similarly, the M^{+14} C32:1 fatty acid partly originates through the same succession of events with the exception that by statistical probability one of the acetyl-CoA units was unlabeled; in addition, in part it comes from uptake of a C18:0/C18:1 fatty acid followed by elongation with ^{13}C -labeled acetyl-CoA units. The relative contributions of the distinct metabolic operations that lead to the same isotope-labeled species cannot be gleaned directly from the labeling pattern of an individual fatty acid. However, this information can potentially be deduced by comparing three sets of observations: (1) acetyl-CoA labeling, (2) the labeling pattern of a fatty acid of interest (a vector of length $N+1$, where N is the number of carbon atoms in the fatty acid, L_{FA} of interest; the entries in the vector correspond to the fractional abundance of each labeled form), and

(3) the labeling patterns of potential source fatty acids. From the labeling patterns of the source fatty acids and acetyl-CoA, it is possible to compute the theoretical labeling patterns of the fatty acid of interest, if it were formed only from that source ($L_{\text{theoretical } i\text{th source of fatty acid}}$). The relative contribution of the different potential sources (α_i) can then be computed by solving for α_i in the equation:

$$L_{\text{FA of interest}} = \sum \alpha_i L_{\text{theoretical } i\text{th source fatty acid}}$$

A limitation is that different sources can be resolved reliably only when they produce sufficiently different theoretical labeling patterns of the fatty acid of interest. Once the sources of different fatty acids are resolved, then fluxes can be globally assigned based on the total consumption of different fatty acids by assimilation into biomass, excretion, and conversion into other fatty acids. We have implemented and begun testing an algorithm incorporating the above concepts, which requires validation and optimization prior to its dissemination.

Impact of activated Ras on fatty acid levels and labeling

Constitutive activation of the Ras signaling pathway through mutations is a common event in various aggressive cancer types, including lung and pancreatic cancer. The Ras pathway is also a target of insulin and it has been shown that oncogenic activation of the pathway causes changes in cellular metabolism that accommodate cellular proliferation.^{26,27} The complete signaling cascade of the Ras pathway has not been elucidated, and accordingly it is possible that the full spectrum of metabolic effects of this pathway have not yet been determined.

In an effort to study the effect of the Ras signaling pathway on fatty metabolism, we compared the levels of fatty acids in immortalized baby mouse kidney epithelial cells with and without expression of activated ^{V12G}Ras (see Figure 5). The cells are isogenic except for the Ras expression and grow at a similar rate in culture, although the Ras-expressing cells lead to much more rapid tumor formation in mice.²¹ As can be seen from Figure 5, most of the long chain fatty acids did not differ significantly between the parental and Ras-expression cell lines. In contrast, almost half of the very-long-chain fatty acids were significantly changed by activated Ras expression, with very-long-chain saturated and monounsaturated fatty acids increased. Thus, the primary effect of Ras on fatty acid metabolism is on very-long-chain fatty acids, whose measurement would have been difficult or impossible with prior analytical methods.

To explore which reactions in fatty acid metabolic pathways were altered to produce the observed changes in very-long-chain fatty acid levels in the two cell lines, the cells were incubated for 72h in culture media containing 10% dialyzed serum and U-¹³C-glucose and U-¹³C-glutamine.

The resulting labeling patterns of the mono-unsaturated very-long-chain fatty acids reveal a marked change in their biosynthetic route across the two cell lines (Figure 6). The labeling pattern of C26:1 from the Ras-expressing cells reveals that approximately 40% of this fatty acid was produced by a single elongation step from an unlabeled C24 fatty acid (the M⁺² peak), whereas only about 10% is produced this way in the parental cell line. Concomitantly, the fractions of the highly ¹³C-labeled C26:1 species are lower in the Ras-expressing cells, indicating decreased *de novo* synthesis. The C28:1 fatty acid in the Ras-expressing cells also appears to be largely the product of elongation of an unlabeled C24 fatty acid, as the labeling pattern shows a high peak (>30%) for the M⁺⁴ form. There is also an increase in the

M⁺² form of this fatty acid, which partly results from the incorporation of both a labeled and an unlabeled 2-carbon unit from acetyl-CoA into a pre-existing C24 fatty acid, as governed by statistical probability (some may also result from the incorporation of a labeled acetyl unit into an unlabeled C26 fatty acid). In a similar fashion, the isotope profiles of the C30:1 and C32:1 fatty acids reveal distinct M⁺⁶ and M⁺⁸ forms, respectively, indicating that they are also produced by elongation of an unlabeled C24 fatty acid. In all cases, the labeling forms associated with elongation of a pre-existing unlabeled C24 fatty acid are distinctly higher in the Ras-expressing cell line. Based on literature, a putative candidate for conducting such elongation is the elongase 4 (ELOVL4) enzyme.²⁵ This enzyme has been shown to be involved in the production of saturated C28 and C30 fatty acids as well as polyunsaturated fatty acids in cultured cells.²⁸ In mice, the knocking out of the *Elovl4* gene resulted in a pronounced depletion of saturated and mono-unsaturated fatty acid having 26 or more carbons in the skin.²⁹ Efforts are ongoing in our labs to explore whether *Elovl4* is the enzyme responsible for the observed metabolic phenotype, a downstream target of the Ras (either direct or indirect), and/or a contributor to the growth of Ras-driven tumors. Potential functions of these very-long-chain saturated and monounsaturated fatty acids include controlling membrane fluidity, trafficking, cell signaling, and/or lipid raft function.^{30,31} The significance of these fatty acid tails to Ras-driven tumor formation remains to be determined.

CONCLUSIONS

Here we present a method for the analysis of fatty acids using liquid chromatography and high resolution mass spectrometry. We consider our approach to be a useful addition to existing fatty acid measurement techniques in that very-long-chain fatty acids can be detected and complete molecular ion labeling data can be obtained for the full scope of long-chain and very-long-chain fatty acids. This allows in-depth investigation into the biochemical processes that contribute to the individual fatty acid pools (uptake, *de novo* synthesis, elongation, and desaturation) in cultured cells. The methods are in principle suitable also to analysis of blood or tissue samples from *in vivo* studies, with the practical utility depending on the extent of isotope labeling that occurs. Here we applied our approach to fatty acids saponified from whole cell lipid extracts; by including additional fractionation steps such as thin layer chromatography, lipid-class specific metabolism could be studied. Adaption of the sample preparation protocol should also make it possible to study the metabolism of ether-linked fatty acids. An important future need is the ability to track labeling into intact lipids, not just fatty acids. Despite extensive progress in lipidomics,^{6,32,33} isotope tracer methods for intact lipids remain limited. As evidenced by a recent study examining the kinetics of incorporation of U-¹³C oleic acid (C18:1) into triglycerides,³⁴ LC-MS holds promise for meeting this important need.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Chromatographic traces of saturated fatty acid standards of varying carbon chain lengths, and (B) of fatty acids with the same carbon chain length but differing degrees of unsaturation. Liquid chromatographic (LC) separation was performed using a C₈ reversed-phase column and a water/methanol gradient containing tributylamine as an ion-pairing agent (see text for details). The LC eluent was ionized with electrospray ionization (negative mode) and subsequently analyzed by a stand-alone orbitrap mass spectrometer.

Figure 2.

Precision and accuracy of ^{13}C natural abundance isotope ratios. For fatty acid standards, experimental isotope ratios arising from natural ^{13}C abundance were assessed through analysis of the M^{+1}/M^0 peak ratios. For each fatty acid, the reported ratio is the average $\pm$ standard deviation across standards injected at 10, 50, 100, 200, 500, 1000, 2000 ng/ml concentrations (10 ng/mL was omitted for the standards with a higher limit of detection). $N = 2$ injections were performed at each concentration. Black bars reflect theoretical values (see main text for equations).

Figure 3. Exemplary data from an isotope labeling experiment with U-¹³C-glucose and U-¹³C-glutamine. **(A)** Base peak chromatogram from saponified total cell lipid extract of immortalized baby mouse kidney epithelial cells (iBMK cells); **(B – D)** Raw mass spectra of C16:0 (palmitate) after **(B)** 0 hours, **(C)** 8 hours, and **(D)** 96 hours of labeling.

Figure 4. Metabolic events contributing to fatty acid ^{13}C -labeling patterns (exemplified for C32:1 fatty acid following 96 h of labeling with uniformly ^{13}C -glucose and ^{13}C -glutamine; the labeling pattern was corrected for natural abundance of ^{13}C and incomplete labeling in the purportedly U- ^{13}C -glucose and U- ^{13}C -glutamine). Fatty acids having more than 16 carbons, exhibit complex labeling patterns that result from multiple biochemical events. A high degree of ^{13}C incorporation (in this case 20–32 labeled carbons) arises from de novo C16:0 synthesis and subsequent elongation. Less extensively labeled forms arise from uptake of unlabeled, shorter precursor fatty acids and their subsequent elongation. The unlabeled form reflects residual fatty acids from before the labeling began (minor fraction) and uptake from serum in the medium (major fraction). Bars indicate mean $\pm$ standard deviation (N=2).

Figure 5.

Peak intensities of (A) long-chain fatty acids (C14–C24) and (B) very-long-chain fatty acids (C26–C36) from immortalized baby mouse kidney epithelial cells with or without expression of activated Ras. The cells were grown to 80% confluence in 6 cm culture dishes in DMEM containing 10% dialyzed serum and unlabeled glucose and glutamine. The extracted and saponified fatty acids were resuspended in 1:1:0.3 chloroform/methanol/water solution to a final concentration of 2 μ l packed cell volume per 1 ml solvent for analysis of the more abundant long-chain fatty acids, and to a 45-fold higher concentration (90 μ l cell volume per 1 mL solvent) for analysis of the less abundant very-long-chain fatty acids, and samples analyzed by LC-MS. Resulting peak heights were corrected for residual differences in total fatty acids injected between the two cell lines by correcting individual peak intensities with the total ion current (TIC) for a given sample. Bars indicate mean $\pm$ SD (N=3). * indicate significant differences in FA levels between parental and activated Ras expressing cells ($p < 0.05$, unpaired two-tailed t-test equal variance, Bonferroni corrected).

Figure 6.

^{13}C -labeling patterns for very-long-chain monounsaturated fatty acids from immortalized baby mouse kidney epithelial cells with and without expression of activated Ras. Cells were maintained for 72 hour in DMEM with $\text{U-}^{13}\text{C}$ -glucose and $\text{U-}^{13}\text{C}$ -glutamine and 10% dialyzed FBS. In the schematic drawing of the fatty acids, * represent ^{13}C atoms. The double bonds are shown at their most likely position but have not been experimentally determined. Bars indicate mean $\pm$ standard deviation (N=2).

Table 1

Retention time, limit of detection (LOD), linearity (of absolute signal intensities), and linear range for individual fatty acid standards.

Fatty acid	RT	LOD (ng/ml)	R ²	Linear range (ng/ml)
C16:0	10.7	80	0.9946	80–2000
C16:1	9.2	4	0.9960	4–2000
C18:0	13.5	70	0.9952	70–2000
C18:1	11.6	1	0.9960	1–2000
C18:2	10.0	10	0.9968	10–2000
C20:0	15.9	20	0.9954	20–2000
C20:1	14.2	10	0.9925	10–2000
C20:3	11.4	1	0.9919	1–2000
C20:4	9.8	1	0.9982	1–2000
C22:0	18.1	2	0.9938	2–2000
C22:1	16.4	150	0.9723	150–2000
C22:4	12.0	1	0.9948	1–2000
C24:0	20.1	15	0.9768	15–2000
C24:1	18.5	25	0.9947	25–2000
C26:0	23.0	4	0.9975	4–2000
C28:0	25.1	5	0.9894	5–2000
C30:0	27.9	5	0.9980	5–2000