Published in final edited form as:

Bioanalysis. 2013 December ; 5(24): 3009–3021. doi:10.4155/bio.13.269.

Human platelets as a platform to monitor metabolic biomarkers using stable isotopes and LC–MS

Sankha S Basu^{1,2}, Eric C Deutsch^{1,3}, Alec A Schmaier¹, David R Lynch^{1,3}, and Ian A Blair^{*},
1,2

¹Department of Pharmacology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

²Centers of Excellence in Environmental Toxicology & Cancer Pharmacology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, USA

³Departments of Neurology & Pediatrics, The Children's Hospital of Philadelphia, & the Perelman School of Medicine at the University of Pennsylvania, PA 19104, USA

Abstract

Background—Intracellular metabolites such as CoA thioesters are modulated in a number of clinical settings. Their accurate measurement from surrogate tissues such as platelets may provide additional information to current serum and urinary biomarkers.

Methods—Freshly isolated platelets from healthy volunteers were treated with rotenone, propionate or isotopically labeled metabolic tracers. Using a recently developed LC–MS-based methodology, absolute changes in short-chain acyl-CoA thioesters were monitored, as well as relative metabolic labeling using isotopomer distribution analysis.

Results—Consistent with *in vitro* experiments, isolated platelets treated with rotenone showed decreased intracellular succinyl-CoA and increased β -hydroxybutyryl-CoA, while propionate treatment resulted in increased propionyl-CoA. In addition, isotopomers of the CoAs were readily detected in platelets treated with the [¹³C]- or [¹³C¹⁵N]-labeled metabolic precursors.

Conclusion—Here, we show that human platelets can provide a powerful *ex vivo* challenge platform with potential clinical diagnostic and biomarker discovery applications.

Mitochondrial dysfunction has been implicated in diabetes [1], heart disease [2], cancer [3], Alzheimer's disease [4], Parkinson's disease [5], autism [6,7], and numerous other neurological and metabolic disorders [8,9]. As a result, there has been a renewed emphasis on the development of clinically applicable biomarkers as well as novel platforms to monitor mitochondrial metabolism [10–12]. Most metabolomic approaches for biomarker discovery have focused on absolute or relative differences in serum and urinary metabolite concentrations [13]. Indeed, such approaches have been critical in the diagnosis of several

© 2013 Future Science Ltd

*Author for correspondence: Tel.: +1 215 573 9880 Fax: +1 215 573 9889 ianblair@mail.med.upenn.edu.

Financial disclosure The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Supplementary data To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/BIO.13.269

metabolic diseases, such as inborn errors of metabolism, where the inheritance of enzyme deficiencies, in expression or activity, results in the buildup of specific metabolites such as organic acids or acyl-carnitines in the serum or urine [14]. Another important series of metabolites includes **CoA** and acyl-CoA thioesters [15,16]. Changes in the intracellular concentration of these metabolites occur in both physiological and pathological settings [17]. Accordingly, the ability to accurately measure various CoA thioesters would be useful in a variety of clinical settings. However, unlike organic acids and acyl-carnitines, acyl-CoA thioesters are not actively secreted and are consequently found in low concentration in body fluids. Therefore, the measurement of these important metabolites requires extractions from tissues or cells for clinical biomarker applications [18,19].

Stable-isotope dilution LC–MS methodology represents the gold standard for measuring many endogenous metabolites [20,21] and represents a powerful, sensitive and specific method to measure CoA thioesters. Due to the limited number of commercially available stable-isotope IS, we recently developed a stable-isotope LC–MS method to generate short-chain CoA IS using stable-isotope labeling by essential nutrients in cell culture (SILEC) [22]. Using these standards, we were able to accurately measure changes in short-chain acyl-CoA thioesters in various *in vitro* settings [23,24]. Murine hepatoma cells (Hepa 1c1c7) treated with propionate demonstrated a dramatic increase in propionyl-CoA and decrease in various other CoA species [22]. Additionally, treatment of several human cell lines with rotenone, a naturally occurring complex I inhibitor, resulted in a dose-dependent decrease in intracellular succinyl-CoA, and a concomitant increase in β -hydroxybutyryl-CoA (BHB-CoA) [25].

While measuring changes in intracellular acyl-CoA levels in cell culture allows characterization of metabolic or toxic insults *in vitro*, the pathological manifestations of many mitochondrial and metabolic diseases are often found in clinically inaccessible cardiac or neuronal tissues [26]. Therefore, clinically accessible surrogate cells or tissues must be used to measure intracellular molecules such as CoA thioesters, in the prospect that they may reflect a perturbed metabolic state, despite the lack of an overt pathological phenotype. In addition to clinical accessibility, another advantage of using viable surrogate cells is an increased ability to perform functional tests rather than the traditional bio-marker approach of monitoring baseline metabolite concentrations. In functional tests, a specific challenge is given, either *in vivo*, such as an oral glucose tolerance test, or *ex vivo*, in which the challenge substrate or toxin is applied to a viable extracted tissue. These types of assays may reveal phenotypic differences between controls and affected individuals that may not be readily discernible in baseline measurements. In addition, since a smaller, controlled amount of cells is being tested, a reduced amount of reagent or testing compound is required for the test as this does not need to be systemically distributed. This is particularly useful for isotopic tracer analysis [27], in which the labeled substrate may be prohibitively expensive, impractical or unsafe to apply *in vivo*.

A useful ***ex vivo* challenge** platform requires a metabolically active, analyte-rich, clinically practical surrogate tissue or cell type. Surrogate cells can come from a wide variety of sources, from mucosal cells and dermal fibroblasts to more clinically practical sources, such as lymphocytes or **platelets** separated from whole blood [28,29]. Preliminary experiments showed relatively low concentrations of CoA thioesters in isolated lymphocytes, whereas the platelet fraction contained much higher intracellular concentrations of these molecules. Due to the abundance of intracellular CoA species, we hypothesized that isolated human platelets could be used as an *ex vivo* platform to monitor metabolic or mitochondrial pathology by measuring changes in intracellular CoA thioester concentrations or changes in relative isotopic labeling when treated with toxic or metabolic challenges. To achieve this, four methodological approaches were tested, as illustrated in Figure 1. In order to assess the

viability of platelets as an *ex vivo* platform and to characterize their metabolic response to mitochondrial toxins, freshly isolated platelets were treated with rotenone, a complex I inhibitor linked to Parkinson's disease in humans and rodents (Figure 1A) [30]. Treatment with propionate was used to assess platelet metabolic activity and viability *ex vivo* with an exogenous substrate (Figure 1B). In these first two approaches, labeled SILEC CoA standards were used as stable-isotope IS to more accurately measure changes in absolute intracellular short-chain acyl-CoA levels in platelets [23]. In the third approach (Figure 1C), various isotopic tracers were applied to isolated platelets to determine whether isotopomers of the extracted acyl-CoA thioesters could be detected [25]. The fourth approach involved the use of whole blood samples to shorten processing times and expand the clinical utility of the method. Whole blood samples were treated with [^{13}C]- or [$^{13}\text{C}^{15}\text{N}$]-labeled metabolic precursors. The platelets were then isolated and extracted, in order to determine whether similar results to those observed with pre-isolated platelets could be obtained. (Figure 1D). For the isotopic tracer experiments, **mass isotopomer distribution** (MID) [31] analysis was performed on the acyl-CoA thioesters to determine the relative isotopic abundance, as a measure of activity in a particular metabolic pathway. Together, these methods present a novel approach of using platelets as an *ex vivo* platform to monitor mitochondrial and metabolic disease.

Materials & methods

Materials

[U- $^{13}\text{C}_6$]-glucose (99%), [1- $^{13}\text{C}_1$]-propionate (99%) and [1,2- $^{13}\text{C}_2$]-acetate (99%) were purchased from Cambridge Isotopes (MA, USA). [U- $^{13}\text{C}_{16}$]-palmitic acid (99%) was purchased from Sigma (MO, USA) and [$^{13}\text{C}^{15}\text{N}_1$]-pantothenic acid calcium, salt (^{13}C 99%, ^{15}N 98%) was purchased from Isosciences (PA, USA). 8.5 ml acid-citrate-dextrose Vacutainer® tubes (whole blood tube w/ anticoagulant, acid-citrate-dextrose Sol A) were purchased from BD Biosciences (REF 364606; NJ, USA). All solvents for LC-MS were Optima grade and purchased from Fisher Scientific (PA, USA).

Isolation & preparation of human platelets

Platelets used in this study were harvested from healthy volunteers at the Children's Hospital of Philadelphia (PA, USA), as part of an ongoing Friedreich's Ataxia Research Alliance study. All subjects provided written, informed consent before participating in study procedures. Briefly, whole blood was drawn from healthy volunteers into 8.5 ml acid-citrate-dextrose Vacutainer tubes. Blood was then transferred to 15 ml conical polypropylene tubes and spun for 15 min at 129 g with no brakes. The upper platelet-rich plasma layer was transferred to a new 15 ml conical tube and spun again at 329 g with no brakes for 15 min. The platelet pellet was resuspended gently in 1 ml Tyrode's solution (139 mM NaCl, 3 mM KCl, 17 mM NaHCO_3 , 12 mM glucose, 3 mM CaCl_2 and 1 mM MgCl_2) and transferred to 1.5 ml microcentrifuge tubes. A 10 μl aliquot was used to determine platelet counts using a Coulter Z1 particle counter (Beckman Coulter).

Extraction of CoA thioesters

Extraction of acyl-CoA thioesters and CoA itself from platelets was performed using minor modifications to previously published methods performed in cell culture [22]. Briefly, purified platelets were pelleted at $1000 \times g$ for 2 min, the supernatant was aspirated, and the pellet was pulse-sonicated for 30 s in 1 ml cold 10% trichloroacetic acid using a sonic dismembrator (Fisher Scientific, MA, USA). For absolute quantitation studies, the trichloroacetic acid lysis solution was spiked with isotopically labeled CoA standards that were generated in murine hepatoma cells using SILEC methodology as previously reported [22]. For isotopic tracer studies, no labeled IS were used [25]. Following sonication, the

lysate was centrifuged at $15000 \times g$ for 5 min. The supernatant was transferred to a fresh tube and the pellet was discarded. Short-chain acyl-CoA species were purified from the sonicated lysate using OasisTM HLB 1 cc (30 mg) SPE columns (Waters, MA, USA) as previously reported [23]. Eluted compounds were dried down under nitrogen and resuspended in 50 μ l 5% 5-sulfosalicylic acid.

LC-MS

Samples were maintained at 4°C in a Leap CTC autosampler (CTC Analytics, Switzerland) during sample batch runs. Injections of 10 μ l were used for LC-MS analysis. Chromatographic separation was performed using a reversed-phase Waters XBridgeTM C18 column (2.1 $\times$ 150 mm, pore size 3 μ m) on an Agilent 1100 HPLC system using a three solvent system: (A) 5 mM ammonium acetate in water; (B) 5 mM ammonium acetate in 95/5 acetonitrile/water (v/v); and (C) 80/20/0.1 (v/v/v) acetonitrile/water/formic acid, with a constant flow rate of 0.2 ml/min. Solvent gradient was performed as follows: 2% B (isocratic) for 1.5 min, 2–20% B (linear gradient) over 3.5 min, 20–100% B (linear gradient) over 0.5 min, 100% B (isocratic) for 8 min, 100% C for 5 min, before equilibration to initial conditions for 5 min. Samples were analyzed using an API 4000TM triple quadrupole mass spectrometer (Applied Biosystems, CA, USA) in the ESI+ mode, and analytes were quantified using Analyst[®] software. The column effluent was diverted to the mass spectrometer from 8 to 13 min and to waste for the remainder of the run. The mass spectrometer operating conditions were as follows: ion spray voltage (5.0 kV), compressed air as curtain gas (15 psi) and nitrogen as nebulizing gas (8 psi), heater (15 psi), and collision-induced dissociation gas (5 psi). The ESI probe temperature was 450°C, the declustering potential was 105 V, the entrance potential was 10 V, the collision energy was 45 eV, and the collision exit potential was 15 V. CoA thioesters were monitored using SRM as previously published [22]. MID analysis was performed by quantifying the M, M+1, M+2, M+3 and M+4 isotopomers (M+5 and M+6 isotopomers were also analyzed in the case of 3-hydroxy-3-methylglutaryl-CoA [HMG-CoA], which contains six carbons), accounting for natural isotopic contribution from the parent compound by using previously described methods [25]. In the case of [¹³C ¹⁵N₁]-pantothenate labeling, isotopic labeling was determined by using the ratios of labeled (M+4) to unlabeled (M) CoA isotopes, as there was negligible (<0.2%) contribution from the unlabeled precursor.

Ex vivo platelet challenges to measure changes in absolute quantitation of CoA thioesters

Ex vivo challenges were performed on resuspended platelets in 1 ml of Tyrode's solution. Briefly, two different challenge paradigms were performed, as described below.

- Rotenone challenge: a 10 mM stock of rotenone was freshly prepared in DMSO. Serial dilutions were made in DMSO from 1 mM down to 100 pM. Platelets from an 8.5 ml blood draw were resuspended in 10 ml of Tyrode's solution and divided into equal 1 ml fractions. 10 μ l of each rotenone dilution and DMSO control were then added to each to generate a dosing curve from 1 pM to 100 μ M in concentration;
- Propionate challenge: a stock of 1 M propionate was prepared in water. 10 μ l of this solution was added to platelets in Tyrode's solution for a final concentration of 10 mM. 10 μ l of water was used as control. Incubations were performed in triplicate. Samples were processed for short-chain acyl-CoA thioesters as described above.

Ex vivo platelet challenges using stable isotope tracers

Stable isotope metabolic tracer studies were performed using two approaches: tracer spiked into Tyrode's solution containing isolated platelets or tracer spiked into whole blood followed by platelet isolation and extraction.

- Isolated platelet study: stocks of [$1-^{13}\text{C}$]-propionate (1 M in water), and [$1,2-^{13}\text{C}_2$]-acetate (1 M in water) and [$^{13}\text{C } ^{15}\text{N}_1$]-pantothenic acid (0.3 mg/ml in water) were freshly prepared. Platelets were isolated as previously described and resuspended in 1 ml Tyrode's buffer. Each tube of platelets was spiked with 10 μl of labeling reagent (1:100 dilution) for a final concentration of 10 mM of propionate, 10 mM (acetate) and 3 mg/l (pantothenate). Platelets were gently mixed by inversion and incubated in the presence of challenge at 37°C for 1 h. After incubation with labeling reagent, platelets were processed as previously described and analyzed for MID;
- Whole blood tracer: stocks of [$1,2-^{13}\text{C}_2$]-acetate (100 mM in water), [$\text{U}-^{13}\text{C}_6$]-D-glucose (100 mg/ml in water), and [$\text{U}-^{13}\text{C}_{16}$]-sodium palmitate (10 mM in EtOH) were freshly prepared. After blood draws were performed, 8 ml of blood was transferred to a fresh 15 ml conical tube and immediately spiked with 80 μl of each tracer stock solution (1:100 dilution) for a final concentration of 1 mM (acetate), 1 mg/ml (glucose), and 100 μM (palmitate). Blood tubes were gently mixed by inversion and incubated at 37°C for 1 h. Samples were processed for short-chain acyl-CoA thioesters as previously described.

Results

Short-chain acyl-CoA extraction from freshly isolated human platelets

To quantify intracellular levels of acyl-CoA thioesters and CoA in platelets, we adapted our previously described LC-MS method to platelets. Platelets were isolated from healthy volunteers and separated by centrifugation and resuspended in Tyrode's solution prior to short-chain acyl-CoA extraction using SILEC methodology. A typical SRM chromatogram showing various CoA species co-eluting with their isotopically labeled standards is presented in Figure 2. Equivalent amounts of SILEC acyl-CoA thioester and CoA IS were also spiked into unlabeled standards to generate a standard curve to quantify absolute levels of acyl-CoA thioesters and CoA in platelets. Collision-induced dissociation coupled with MS/MS analysis resulted in a product ion spectrum with the characteristic neutral loss of 507 u (Supplementary Figure 1).

Rotenone challenge

To determine whether platelets can be used as an *ex vivo* platform for toxicological challenges, rotenone, a naturally occurring pesticide and potent complex I inhibitor, was applied to platelets isolated from whole blood. Platelets were divided into equal fractions and treated with increasing doses of rotenone for 1 h, followed by short-chain acyl-CoA thioester analysis (Figure 3). Rotenone decreased intracellular succinyl-CoA levels and increased BHB-CoA levels in a dose-dependent manner, both with an IC_{50} value of <100 nM (Figure 3A & B), consistent with the effect observed in various human cell lines, including SH-SY5Y, HepG2 and H358 [25]. The ratio of BHB-CoA:succinyl-CoA is plotted as a function of rotenone dose, demonstrating a likely relationship between these processes (Figure 3C). These data show that platelets can be used as an *ex vivo* toxicological model, and can potentially be used to evaluate the effect of mitochondrial toxins in different disease states.

Propionate challenge

We have previously shown that treatment of murine hepatoma cells with propionate results in an increase of propionyl-CoA and a decrease of several other CoA species. To confirm that platelets were a comparable *ex vivo* platform, freshly isolated human platelets were treated with 10 mM propionate in Tyrode's solution. After 30 min, propionyl-CoA thioester levels increased tenfold, while concentrations of the acetyl-CoA and succinyl-CoA thioesters decreased significantly (Figure 4), similar to *in vitro* experiments in Hepa 1c1c7 cells [22]. Therefore, this propionate challenge demonstrated that platelets can be used in an *ex vivo* platform to monitor the uptake and incorporation of fatty acids into anabolic and catabolic pathways. Furthermore, the approach can be readily extended to include other fatty acids or nutrients.

Isotopic tracer challenge in isolated platelets

One of the most powerful LC-MS applications in metabolism studies is the use of isotopically labeled metabolic tracers to monitor flux through metabolic pathways *in vitro* as well as *in vivo* [27]. This methodology was applied to platelets isolated from three different subjects. The platelets were treated with [^{13}C]- or [$^{13}\text{C}^{15}\text{N}$]-labeled metabolic precursors tracers for 1 h, followed by the extraction of acyl-CoA thioesters as described above. MID analysis was performed to determine the isotopic abundance of each of the species and subtract the contribution in the labeled channels from the natural isotopic envelope. First, [$^{13}\text{C}_3\ ^{15}\text{N}_1$]-pantothenate (1 mg/l blood) was readily incorporated into each of the analyzed acyl-CoA thioesters, although at different percentages, suggesting different rates of turnover for each of them (Figure 5). The greater increase in [$^{13}\text{C}_3\ ^{15}\text{N}_1$]-pantothenate labeling in certain acyl-CoA thioesters (e.g., HMG-CoA) indicated that a larger portion of such metabolites was synthesized from newly formed CoA. Additionally, isolated platelets were incubated with [1,2- $^{13}\text{C}_2$]-acetate or [1- $^{13}\text{C}_1$]-propionate for 1 h (Figure 6). For acetate labeling, >50% of both acetyl-CoA and succinyl-CoA was labeled (M+2). BHB-CoA demonstrated significant labeling in M+2 and M+4 isotopomers (but not M+1 and M+3), and HMG-CoA were labeled as the M+2, M+4 and M+6 (not M+1, M+3 or M+5) species, consistent with incorporation of two carbon units from acetate. In addition, increased M+3 and M+4 labeling of succinyl-CoA was indicative of multiple turns of the Krebs cycle. For propionate labeling, [1- $^{13}\text{C}_1$]-propionate was incorporated into [1- $^{13}\text{C}_1$]-propionyl-CoA (M+1) and also into succinyl-CoA (M+1), with approximately 30% of the total succinyl-CoA being labeled through this anaplerotic pathway. Therefore, propionate could potentially be used as an anaplerotic biomarker by analyzing its incorporation into succinyl-CoA. Furthermore, the lack of labeling on CoA itself with acetate and propionate confirmed that the isotopic labeling had occurred specifically on the acyl moiety.

Isotopic tracer analysis in whole blood

To determine whether labeling could be performed in whole blood, labeling was evaluated with [1,2- $^{13}\text{C}_2$]-acetate at two different points in the protocol: directly into whole blood (pre-centrifugation) or after platelet isolation (post-centrifugation). Similar labeling efficiency was observed between the two labeling methods (Supplementary Figure 2). [1,2- $^{13}\text{C}_2$]-acetate showed very rich labeling of various short-chain acyl-CoA thioester species and was illustrative from a proof-of-concept standpoint. However, we sought to measure the relative activity of various catabolic pathways in platelets by using precursors that were further upstream. Therefore, [U- $^{13}\text{C}_6$]-glucose was used to monitor glycolysis, and [U- $^{13}\text{C}_{16}$]-palmitate was used to monitor fatty acid oxidation-derived carbon atoms (Figure 7). Interestingly, there was minimal glucose incorporation into any of the CoA species (including acetyl-CoA), whereas approximately 20% of the acetyl-CoA thioester pool (M+2) was derived from palmitate when given at a comparable concentration, suggesting a

preference for fatty acid oxidation over glycolysis as a source for acetyl-CoA under our conditions. In order to address concerns that the lack of glucose labeling was an artifact of spiking samples into whole blood (and potentially diluting the relative level of labeled glucose), the same experiments were performed in Tyrode's solution containing [U-¹³C₆]-glucose, which again showed that there was minimal labeling (<3%) in acetyl-CoA.

Discussion

This study has revealed that freshly isolated human platelets can be used as an *ex vivo* metabolic platform for measuring changes in several acyl-CoA thioesters. Therefore, it was demonstrated that treatment with mitochondrial toxins, such as rotenone, in isolated platelets recapitulated the pheno-type observed with immortalized cell lines. This not only further strengthens our original findings for rotenone-mediated changes in intracellular acyl-CoA species (particularly succinyl-CoA and BHB-CoA) [25], but also demonstrated that platelets provide a viable surrogate tissue to study mitochondrial or metabolic dysfunction. In addition, the metabolic changes observed in platelets were essentially identical to those observed previously in SH-SY5Y, HepG2 and H358 cell lines. This confirms that the changes in acyl-CoA thioester levels were not a cancer-specific phenomenon, but rather suggests a general physiological metabolic rearrangement in response to rotenone. Similar approaches could be used to evaluate susceptibility to other suspected environmental mitochondrial toxins that may play a role in neurodegenerative disease.

There is mounting evidence that idiosyncratic adverse drug reactions might involve metabolic and mitochondrial toxicity [32]. The action of drugs and their metabolites on platelet metabolism offers a new way to explore this possibility in more detail. An advantage of an *ex vivo*, rather than an *in vivo* approach, is the possibility of testing a wide variety of potential drug candidates and their metabolites, new chemicals or toxins, which would be unsafe to use *in vivo* [33]. In addition, this approach could also be applied to diseases such as Friedreich's ataxia, which are genetic in origin. There are currently no clinically useful biomarkers of the disease and recent studies have suggested that metabolic dysfunction could play an important pathogenic role [34,35]. While most of the clinical manifestations of Friedreich's ataxia occur in clinically inaccessible tissues [36], such as in cardiomyocytes in the myometrium and spinocerebellar pathways, by using carefully selected agents or stressors in platelets, potential phenotypic differences might be uncovered, allowing for biochemical assessment of potential treatment [37].

In addition, it was shown that exogenous substrates, such as propionate, are readily taken up and metabolized by isolated platelets. The increase in propionyl-CoA and decrease in other acyl-CoA species after propionate treatment mirrored changes observed previously in murine hepatoma cells, demonstrating that platelets are not only a viable active metabolic platform, but may also serve as a potential surrogate metabolic tissue. This could then characterize novel biomarkers or even provide a diagnostic tool for inborn errors of metabolism [38]. This latter possibility would be revealed by insufficient downstream products or a buildup of precursors resulting from an enzymatic deficiency.

Finally, the use of several isotope tracers has revealed that in as little as an hour, several different labeled substrates could be incorporated and readily detected in the short-chain acyl-CoA thioesters extracted from freshly isolated platelets. However, unlike unlabeled propionate, the exact carbons from each isotopically labeled substrate could be readily traced through various metabolic pathways. These data could then be used to determine the metabolic flux in a particular pathway using previously described methods [25,27]. This is perhaps the most powerful application of the present methodology and could be used to monitor changes in a number of different clinical settings. The ability to collect, wash and

re-suspend platelets *ex vivo* prior to testing affords increased standardization and reduces variability that may arise from physiological factors, such as diet, recent medical procedures, and nutrient content in the blood. Conversely, platelet isolation increases processing time and decreases clinical applicability. Therefore, we also tested whether whole blood could be spiked with an isotopic tracer, such as [1,2-¹³C₂]-acetate and, indeed, similar isotopomer incorporation was observed in the measured acyl-CoA thioesters in as little as a 30 min after the blood was collected. Accordingly, standardized tubes containing isotopic tracers could be manufactured and used to generate standardized *ex vivo* functional tests, using any of the numerous metabolic tracers currently available or being developed. Nonetheless, the proper tracer, time of application and sample handling techniques need to be optimized for each disease. Although radioactive tracers, such as glutamine, have previously been used for platelet ana lysis in a number of different neurodegenerative settings, these methods have largely focused on total uptake rather than tracing the fate of the labeled atoms [39]. Stable isotope tracers have been used in lymphocytes in various clinical settings as well [40]. However, in our preliminary ana lysis, compared with other harvested cells, such as buccal cells or lymphocytes, platelets had significantly higher levels of intracellular short-chain acyl-CoA species, probably reflecting a higher mitochondrial content. This allows smaller sample volumes and, consequently, a greater number of functional tests that might be performed using the same blood draw. Unfortunately, platelets cannot currently be cultured for longer incubations, which is an advantage for dermal fibroblasts or lymphocytes. Furthermore, frozen historical samples will probably not be applicable to this new method, since fresh metabolically active platelets are required. On the other hand, data from *ex vivo* platelet challenges could be combined with similar experiments with cultured lymphocyte or fibroblasts to generate a richer characterization of metabolic pathways. This would be important from a diagnostic standpoint and through an improvement in the mechanistic understanding of the role of metabolic defects in various clinical diseases.

Conclusion

Freshly collected human platelets provide a readily accessible tissue that maintains its phenotype *ex vivo*. Any subtle phenotypic differences, such as alterations in mitochondrial metabolism, will be reflected by changes to metabolite levels, which can be readily quantified using the high precision and accuracy of SILEC methodology. This can then provide clues to further understanding any mitochondrial dysfunction that might be present *in vivo*. Furthermore, by conducting isotopomer analysis of metabolites arising from stable isotopically labeled precursors, it is possible to obtain additional information on disruptions to particular metabolic pathways. Consequently, the use of functional metabolic tests, rather than absolute metabolic measurements, allows for richer and more dynamic data sets to be generated, in comparison with classical biomarker approaches of analyzing metabolites in plasma or urine. The *ex vivo* platelet applications described in this report represent not only a novel surrogate tissue to study metabolism, but also provide a methodological advance in biomarker development.

Future perspective

It is anticipated that the new platelet platform will reveal novel biomarkers for genetic diseases such as Friedreich's ataxia and diseases with both genetic and environmental components, such as Parkinson's disease. It should be possible to conduct *ex vivo* biomarker studies using platelets obtained from patients with both of these diseases. Initially, both unlabeled and labeled isotopomer approaches should be conducted. Once the relevant disease biomarkers have been identified, it will be possible to test the efficiency with which new treatment strategies can inhibit their formation as well as allowing the long-term effectiveness of new treatments to be monitored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by the NIH through grant number P30ES013508 and training grant number 5T32HL007439, as well as a grant from the Friedreich ataxia research alliance.

Glossary

CoA	Ubiquitous intracellular thiol common to all forms of life that serves critical metabolic and detoxification functions. It is found predominantly as a thioester bound to short-, medium- or long-chain fatty acids.
Stable-isotope dilution LC-MS	HPLC separation when coupled with MS/MS and lysis using stable-isotope IS provides a robust, sensitive and specific platform for bioanalysis of endogenous compounds.
Stable-isotope labeling by essential nutrients in cell culture	Using a method similar to stable-isotope labeling by amino acids in cell culture, stable-isotope labeling by essential nutrients in cell culture methodology replaces labeled amino acids with heavy labeled essential nutrients, such as [$^{13}\text{C}_3^{15}\text{N}_1$]-pantothenic acid, for the biosynthetic generation of isotopically labeled CoA derivatives for use as MS IS.
<i>Ex vivo</i> challenge	Application of exogenous substrates or stimuli, including labeled or unlabeled nutrients, toxins or pharmaceuticals, to viable cell or tissue samples isolated from subjects in order to characterize a particular physiological or pathological process. This methodology avoids assays that have practical or ethical limitations if performed <i>in vivo</i> .
Platelets	Also known as 'thrombocytes'; cell fragments lacking nuclear components derived from megakaryocytes that play a critical role in hemostasis. In this study, human platelets isolated from whole blood are utilized as an <i>ex vivo</i> platform to analyze metabolism in diseases that affect clinically inaccessible tissues.
Mass isotopomer distribution	Analysis that involves the measuring of relative isotopic abundance of endogenous compounds by MS in cells or tissues labeled with isotopically labeled metabolic tracers to determine the relative activity of a metabolic enzyme or pathway.

References

Papers of special note have been highlighted as:

■ of interest

■ of considerable interest

1. Patti ME, Corvera S. The role of mitochondria in the pathogenesis of Type 2 diabetes. *Endocr. Rev.* 2010; 31(3):364–395. [PubMed: 20156986]
2. Griffiths EJ. Mitochondria and heart disease. *Adv. Exp. Med. Biol.* 2012; 942:249–267. [PubMed: 22399426]

3. Wallace DC. Mitochondria and cancer. *Nat. Rev. Cancer*. 2012; 12(10):685–698. [PubMed: 23001348]
4. Swerdlow RH. Brain aging, Alzheimer's disease, and mitochondria. *Biochim. Biophys. Acta*. 2011; 1812(12):1630–1639. [PubMed: 21920438]
5. Schapira AH. Targeting mitochondria for neuroprotection in Parkinson's disease. *Antioxid. Redox Signal*. 2012; 16(9):965–973. [PubMed: 22229791]
6. Dhillon S, Hellings JA, Butler MG. Genetics and mitochondrial abnormalities in autism spectrum disorders: a review. *Curr. Genomics*. 2011; 12(5):322–332. [PubMed: 22294875]
7. Palmieri L, Persico AM. Mitochondrial dysfunction in autism spectrum disorders: cause or effect? *Biochim. Biophys. Acta*. 2010; 1797(6–7):1130–1137. [PubMed: 20441769]
8. Mancuso C, Scapagini G, Curro D, Giuffrida Stella AM, De MC, Butterfield DA, et al. Mitochondrial dysfunction, free radical generation and cellular stress response in neurodegenerative disorders. *Front Biosci*. 2007; 12:1107–1123. [PubMed: 17127365]
9. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* 2005; 39:359–407. [PubMed: 16285865]
10. Sampey BP, Freerman AJ, Zhang J, et al. Metabolomic profiling reveals mitochondrial-derived lipid biomarkers that drive obesity-associated inflammation. *PLoS ONE*. 2012; 7(6):e38812. [PubMed: 22701716]
11. Suomalainen A. Biomarkers for mitochondrial respiratory chain disorders. *J. Inherit. Metab. Dis*. 2011; 34(2):277–282. [PubMed: 20941643]
12. Filiou MD, Zhang Y, Teplytska L, et al. Proteomics and metabolomics analysis of a trait anxiety mouse model reveals divergent mitochondrial pathways. *Biol. Psychiatry*. 2011; 70(11):1074–1082. [PubMed: 21791337]
13. Kristal BS, Shurubor YI, Kaddurah-Daouk R, Matson WR. Metabolomics in the study of aging and caloric restriction. *Methods Mol. Biol.* 2007; 371:393–409. [PubMed: 17634593]
14. Jones PM, Bennett MJ. Urine organic acid analysis for inherited metabolic disease using gas chromatography-mass spectrometry. *Methods Mol. Biol.* 2010; 603:423–431. [PubMed: 20077094]
15. Spry C, Kirk K, Saliba KJ. Coenzyme A biosynthesis: an antimicrobial drug target. *FEMS Microbiol. Rev.* 2008; 32(1):56–106. [PubMed: 18173393]
16. Ellis JM, Frahm JL, Li LO, Coleman RA. Acyl-coenzyme A synthetases in metabolic control. *Curr. Opin. Lipidol.* 2010; 21(3):212–217. [PubMed: 20480548]
17. Haynes CA, Allegood JC, Sims K, Wang EW, Sullards MC, Merrill AH Jr. Quantitation of fatty acyl-coenzyme As in mammalian cells by liquid chromatography- electrospray ionization tandem mass spectrometry. *J. Lipid Res.* 2008; 49(5):1113–1125. [PubMed: 18287618] ■Describes the use of LC–MS for quantification of acyl-CoA thioesters.
18. Palladino AA, Chen J, Kallish S, Stanley CA, Bennett MJ. Measurement of tissue acyl-CoAs using flow-injection tandem mass spectrometry: acyl-CoA profiles in short-chain fatty acid oxidation defects. *Mol. Genet. Metab.* 2012; 107(4):679–683. [PubMed: 23117082] ■Describes the use of LC–MS for quantification of acyl-CoA thioesters in tissue samples.
19. Magnes C, Suppan M, Pieber TR, et al. Validated comprehensive analytical method for quantification of coenzyme A activated compounds in biological tissues by online solid-phase extraction LC-MS/MS. *Anal. Chem.* 2008; 80(15):5736–5742. [PubMed: 18613647] ■Describes the use of LC–MS for quantification of acyl-CoA thioesters in tissue samples.
20. Ciccimaro E, Blair IA. Stable-isotope dilution LC-MS for quantitative biomarker analysis. *Bioanalysis*. 2010; 2(2):311–341.
21. Geiger T, Wisniewski JR, Cox J, et al. Use of stable isotope labeling by amino acids in cell culture as a spike-in standard in quantitative proteomics. *Nat. Protoc.* 2011; 6(2):147–157. [PubMed: 21293456]
22. Basu SS, Mesaros C, Gelhaus SL, Blair IA. Stable isotope labeling by essential nutrients in cell culture for the preparation of labeled coenzyme A and its thioesters. *Anal. Chem.* 2011; 83(4):1363–1369. [PubMed: 21268609] ■Description of the underlying concepts for conducting stable-isotope labeling by essential nutrients in cell culture.

23. Basu SS, Blair IA. SILEC: a protocol for generating and using isotopically labeled coenzyme A mass spectrometry standards. *Nat. Protoc.* 2012; 7(1):1–12. [PubMed: 22157971] ■ Detailed methods for conducting the stable-isotope labeling of CoA and acyl-CoA thioesters.
24. Rangiah K, Tippornwong M, Sangar V, et al. Differential secreted proteome approach in murine model for candidate biomarker discovery in colon cancer. *J. Proteome Res.* 2009; 8(11):5153–5164. [PubMed: 19769411]
25. Basu SS, Blair IA. Rotenone-mediated changes in intracellular coenzyme A thioester levels: implications for mitochondrial dysfunction. *Chem. Res. Toxicol.* 2011; 24(10):1630–1632. [PubMed: 21950265] ■ First description of decreased succinyl-CoA biosynthesis after inhibition of complex I by rotenone.
26. Tremolizzo L, Sala G, Zoia CP, Ferrarese C. Assessing glutamatergic function and dysfunction in peripheral tissues. *Curr. Med. Chem.* 2012; 19(9):1310–1315. [PubMed: 22304709]
27. Metallo CM, Walther JL, Stephanopoulos G. Evaluation of ¹³C isotopic tracers for metabolic flux analysis in mammalian cells. *J. Biotechnol.* 2009; 144(3):167–174. [PubMed: 19622376]
28. Deutsch EC, Santani AB, Perlman SL, et al. A rapid, noninvasive immunoassay for frataxin: utility in assessment of Friedreich ataxia. *Mol. Genet. Metab.* 2010; 101(2–3):238–245. [PubMed: 20675166] ■ Example of biomarker analysis for the genetic neurodegenerative disease of Friedreich ataxia.
29. Selak MA, Lyver E, Micklow E, et al. Blood cells from Friedreich ataxia patients harbor frataxin deficiency without a loss of mitochondrial function. *Mitochondrion.* 2011; 11(2):342–350. [PubMed: 21147271]
30. Tanner CM, Kamel F, Ross GW, et al. Rotenone, paraquat, and Parkinson's disease. *Environ. Health Perspect.* 2011; 119(6):866–872. [PubMed: 21269927] ■ Definitive study showing the link between paraquat exposure and Parkinson's disease.
31. Metallo CM, Gameiro PA, Bell EL, et al. Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. *Nature.* 2012; 481(7381):380–384. [PubMed: 22101433]
32. Thompson RA, Isin EM, Li Y, et al. *In vitro* approach to assess the potential for risk of idiosyncratic adverse reactions caused by candidate drugs. *Chem. Res. Toxicol.* 2012; 25(8):1616–1632. [PubMed: 22646477]
33. Rockett JC, Burczynski ME, Fornace AJ, Herrmann PC, Krawetz SA, Dix DJ. Surrogate tissue analysis: monitoring toxicant exposure and health status of inaccessible tissues through the analysis of accessible tissues and cells. *Toxicol. Appl. Pharmacol.* 2004; 194(2):189–199. [PubMed: 14736499] ■ Use of surrogate tissues for conducting biomarker analysis.
34. Santos R, Lefevre S, Sliwa D, Seguin A, Camadro JM, Lesuisse E. Friedreich ataxia: molecular mechanisms, redox considerations, and therapeutic opportunities. *Antioxid. Redox Signal.* 2010; 13(5):651–690. [PubMed: 20156111]
35. Sutak R, Xu X, Whitnall M, Kashem MA, Vyoral D, Richardson DR. Proteomic analysis of hearts from frataxin knockout mice: marked rearrangement of energy metabolism, a response to cellular stress and altered expression of proteins involved in cell structure, motility and metabolism. *Proteomics.* 2008; 8(8):1731–1741. [PubMed: 18340635]
36. Lynch DR, Deutsch EC, Wilson RB, Tennekoon G. Unanswered questions in Friedreich ataxia. *J. Child Neurol.* 2012; 27(9):1223–1229. [PubMed: 22832776]
37. Schmucker S, Puccio H. Understanding the molecular mechanisms of Friedreich's ataxia to develop therapeutic approaches. *Hum. Mol. Genet.* 2010; 19(R1):R103–R110. [PubMed: 20413654]
38. Mitchell GA, Gauthier N, Lesimple A, Wang SP, Mamer O, Qureshi I. Hereditary and acquired diseases of acyl-coenzyme A metabolism. *Mol. Genet. Metab.* 2008; 94(1):4–15. [PubMed: 18337138] ■ Convincingly demonstrates the utility of acyl-CoA thioesters as metabolic biomarkers of disease.
39. Vaccaro M, Riva C, Tremolizzo L, et al. Platelet glutamate uptake and release in migraine with and without aura. *Cephalalgia.* 2007; 27(1):35–40. [PubMed: 17212681] ■ Earlier study that also advocated the use of human platelets as surrogates for *in vivo* biomarker analysis.
40. Le A, Lane AN, Hamaker M, et al. Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. *Cell Metab.* 2012; 15(1):110–121. [PubMed: 22225880] ■

An excellent study showing how glutamine can replace glucose as a precursor for the generation of α -keto-glutarate in cancer cells.

Executive summary

Materials & methods

- Platelets from healthy volunteers were isolated and treated with either rotenone, a mitochondrial complex I inhibitor, or propionate, a short-chain fatty acid.
- In addition, isolated platelets were treated with isotopically labeled metabolic tracers and relative incorporation of the labeled atoms was determined in the extracted short chain acyl-CoA thioesters and CoA itself.

Results

- Platelets treated for 1 h with rotenone had decreased intracellular succinyl-CoA and increased β -hydroxybutyryl-CoA.
- Propionate treatment resulted in increased synthesis of intracellular propionyl-CoA.
- Isotopomers of the CoAs were readily detected in platelets treated with the [^{13}C]- or [$^{13}\text{C}^{15}\text{N}$]-labeled metabolic precursors either before or after isolation of the platelets.
- Human platelets provide an *ex vivo* challenge platform for the discovery of potential mitochondrial metabolic biomarkers.

Figure 1. Four different *ex vivo* challenges schemes in isolated human platelets

Platelets are purified from whole blood using differential centrifugation and treated with either (A) a toxin, or (B) a metabolic substrate, followed by absolute quantitative analysis using labeled IS. Metabolic analysis using stable-isotope tracers applied either (C) after or (D) before platelet isolation.

MID: Mass isotopomer distribution; PRP: Platelet-rich plasma; SILEC: Stable-isotope labeling with essential nutrients in cell culture.

Figure 2. Representative LC-SRM/MS chromatogram of short-chain acyl-CoA thioesters extracted from untreated platelets
 BHB-CoA: β -hydroxybutyryl-CoA; CoASH: Reduced CoA; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.

Figure 3. Dose-dependent effect of rotenone on succinyl-CoA and β-hydroxybutyryl-CoA in isolated human platelets

Platelets from one healthy subject were isolated, washed and resuspended in Tyrode's solution containing an increasing concentration of rotenone for 30 min. Following incubation, platelets were pelleted and processed for short-chain acyl-CoA concentrations. A small aliquot was taken after resuspension to measure platelet concentration. (A) and (B) demonstrate intracellular platelet BHB-CoA and succinyl-CoA levels, respectively; (C) demonstrates the ratio of BHB-CoA to succinyl-CoA.

BHB-CoA: β-hydroxybutyryl-CoA.

Figure 4. Effect of propionate treatment on selected CoA-thioesters in isolated human platelets Platelets from a healthy subject were isolated, washed and resuspended in either Tyrode's solution or Tyrode's solution containing 10 mM propionate for 3 min. Following incubation, platelets were pelleted and processed for short chain acyl-CoA concentrations using stable-isotope labeling by essential nutrients in cell culture methodology. A small aliquot was taken after resuspension to measure platelet concentration. Error bars show SEMs for triplicate determinations.
* $p < 0.005$.

Figure 5. Relative isotopic labeling of pantetheine backbone of various CoA derivatives
Isolated platelets were treated for 1 h with $[^{13}\text{C}_3\ ^{15}\text{N}_1]$ -pantothenate, which was incorporated into intracellular CoASH and acyl-CoA species using a series of five enzymatic steps. Short-chain acyl-CoA thioesters were then extracted from platelets and analyzed for relative isotopic labeling by determining the relative concentration of labeled (M+4) to unlabeled (M) isotopomer. Error bars show SEMs for triplicate determinations. BHB-CoA: β -hydroxybutyryl-CoA; CoASH: Reduced CoA; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.

Figure 6. Mass isotopomer distribution of selected CoA thioesters extracted from freshly isolated human platelets treated with Tyrode's solution containing no isotopic tracers (unlabeled), [1- $^{13}\text{C}_1$]-propionate (10 mM) or [1,2- $^{13}\text{C}_2$]-acetate (10 mM)

Following incubation, isotopic distribution of selected CoA species was determined using mass isotopomer distribution analysis. Error bars show SEMs for triplicate determinations. BHB-CoA: β -hydroxybutyryl-CoA; CoASH: Reduced CoA; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.

Figure 7. Mass isotopomer distribution of selected CoA thioesters extracted from isolated human platelets treated in whole blood with no isotopic tracer (unlabeled), [U- $^{13}\text{C}_6$]-glucose (1 mg/ml) or [U- $^{13}\text{C}_{16}$]-palmitate (100 μM)

Following incubation, isotopic distribution of selected CoA species was determined using mass isotopomer distribution analysis. Error bars show SEMs for triplicate determinations. BHB-CoA: β -hydroxybutyryl-CoA; CoASH: Reduced CoA; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA.