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Review

The use of stable isotopes in the study of human pathophysiology

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ABSTRACT

The growing prevalence of metabolic diseases including fatty liver disease and Type 2 diabetes has increased the emphasis on understanding metabolism at the mechanistic level and how it is perturbed in disease. Metabolomics is a continually expanding field that seeks to measure metabolites in biological systems during a physiological stimulus or a genetic alteration. Typically, metabolomics studies provide total pool sizes of metabolites rather than dynamic flux measurements. More recently there has been a resurgence in approaches that use stable isotopes (e.g. ^{2}H and ^{13}C) for the unambiguous tracking of individual atoms through compartmentalised metabolic networks in humans to determine underlying mechanisms. This is known as metabolic flux analysis and enables the capture of a dynamic picture of the metabolome and its interactions with the genome and proteome. In this review, we describe current approaches using stable isotope labelling in the field of metabolomics and provide examples of studies that led to an improved understanding of glucose, fatty acid and amino acid metabolism in humans, particularly in relation to metabolic disease. Examples include the use of stable isotopes of glucose to study tumour bioenergetics as well as brain metabolism during traumatic brain injury. Lipid tracers have also been used to measure non-esterified fatty acid production whilst amino acid tracers have been used to study the rate of protein digestion on whole body postprandial protein metabolism. In addition, we illustrate the use of stable isotopes for measuring flux in human physiology by providing examples of breath tests to measure insulin resistance and gastric emptying rates.

1. Introduction

1.1. A brief overview of the use of stable isotopes in metabolomics and fluxomics

Metabolomics, the quantification of the complete metabolite complement within a cell, tissue or organism (Goodacre et al., 2004), involves the detection of low molecular weight molecules, achieved using analytical platforms such as Mass Spectrometry (MS) or Nuclear Magnetic Resonance (NMR) Spectroscopy. Historically, most studies have focused on total pool size changes rather than dynamic changes in flux resulting from changes in enzyme activity or cell import and export (Carnicer et al., 2012; Wentzel et al., 2012; Lu et al., 2015). However, metabolite levels are most probably the result of an elaborate network of interconnected pathways and the fluxes through these pathways, which are not necessarily delineated by simple metabolite quantification (You et al., 2014). Furthermore, better understanding flux changes in disease should provide a more detailed mechanistic picture of the pathophysiology.

Various definitions have been proposed for the term 'fluxomics', however, herein it is the 'measurement of the wide set of reaction fluxes within a cell, organ or organism, and reflects a unique dynamic picture of the phenotype' (Cascante and Marin, 2008). Metabolic flux-profiling captures information regarding the metabolome and its functional interactions with the proteome and the genome (Fig. 1). Over the past four decades, researchers have engaged with the use of stable isotopes as mass labels for MS-based detection or making use of the spin properties of nuclei such as ^{13}C and ^{2}D to detect these labels by NMR spectroscopy (Kleckner and Foster, 2012; Beckonert et al., 2007). Collectively, the use of stable isotopes has improved pathway elucidation as well as metabolite annotation and quantification. The combination of fluxome and metabolome can provide a complete picture of the pathophysiology of a cell or an organism at the metabolic level (Sauer, 2006).

However, most flux-based analyses require the direct sampling of cells or tissues to investigate the rate of labelling of metabolites across a time course, particularly when relying on mass spectrometry-based tools. While suitable for *in vitro* cell culture-based experiments, this

Abbreviations: ALS, amyotrophic lateral sclerosis; CVD, cardiovascular disease; GBT, glucose breath test; GC–MS, gas chromatography mass spectrometry; GE, gastric emptying; IRMS, isotope ratio mass spectrometrym; MS, mass spectrometry; NAFLD, non-alcoholic fatty liver disease; NMR, nuclear magnetic resonance; NEFA, non-esterified fatty acid; TCA, tricarboxylic acid; T2DM, type 2 diabetes mellitus

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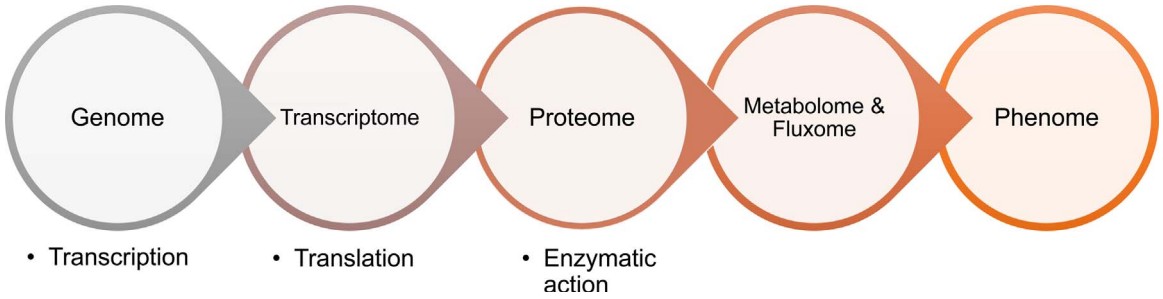

Fig. 1. A linear hierarchal organisation from genome to phenome. The diagram represents the flow of information from the genome to the transcriptome, proteome, and metabolome/fluxome. Each level of organisation may depend on another, and interactions between levels dynamically determines the trait of an organism.

poses a challenge for *in vivo* animal studies, particularly for human physiology. Furthermore, in practice, studying human physiology is challenging as the number of ethical and physiological stresses, such as exercise, nutritional interventions, isotope infusions and drug interventions, available for use in investigations of healthy humans is particularly restricted.

The growing prevalence of obesity and Type 2 diabetes mellitus (T2DM) has increased the emphasis on understanding the regulation of glucose, lipid and protein uptake and metabolism. Glucose and fatty acid metabolism are in part mediated by insulin and in turn excess consumption of both types of substrate mediate insulin resistance (Turner et al., 2014; Cushman et al., 1978). Abnormalities in macronutrient metabolism are associated with T2DM, non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD) (Umpleby, 2015). The concentration of metabolites in biological fluids may provide insights into metabolic irregularities and this approach has been widely used in metabolic disease research (Eiden et al., 2015; Soininen et al., 2009; Orešič et al., 2016). However, it is often challenging to understand what these pool size changes mean in terms of the underlying mechanism(s) of the disease as they do not necessarily provide information about which pathway fluxes are altered. The concentration of a metabolite is dependent on its local synthesis, absorption from dietary sources, release to circulation and degradation (Umpleby, 2015), further complicating its interpretation. To gain an understanding of the regulation of metabolite species, we must be able to quantify fluxes to understand why a change has occurred. This is achieved by labelling the metabolite of interest with a stable isotope, enabling one to 'chase' the label, following and determining its ultimate metabolic fate. This directly measures alterations in flux rather than inferring fluctuations in total pool sizes, and can bring one closer to the causal changes in regulation (Zamboni et al., 2009; Crown et al., 2012; Wittmann, 2002).

1.2. Common nucleotopes used as isotopic tracers

Isotopes are species of an element with the same atomic number but different mass numbers, through the addition of one or more neutrons within the nucleus of an atom. Isotopes can either be radioactive (radio-isotopes) or non-radioactive (stable isotopes) and are used to measure the changes of the major naturally occurring isotope. Exposure of humans to radio-isotopes can be potentially harmful, and there has been a move away from these wherever possible in biology and medicine. Stable isotopes, including ^{15}N, ^{13}C, ^{18}O, and ^{2}H, are thus more suitable for human research. Stable isotopes are, overall, chemically similar to the major isotope found in biological systems (tracee) when incorporated into endogenous compounds. For this reason, they can be used as isotopic tracers to follow a given metabolic pathway, without interfering in the reactions of that pathway. A notable exception to this is ^{2}H, deuterium, which has the largest kinetic isotope effect of any stable isotope. This arises from the fact that, when compared to the mass increase from ^{12}C to ^{13}C (approximately 8%), the mass of deuterium is effectively double that of hydrogen, and so reaction rates in biological systems are significantly altered compared with the dominant isotope.

Elements with one or more isotopes have a naturally occurring distribution abundance (for example, carbon-12 and carbon-13 have a natural abundance of 98.9% and 1.1% respectively). Therefore, molecules that contain one or more of those isotopes naturally also have a specific distribution pattern with different masses. Typically, the most abundant form of a molecule is labelled as M + 0 and is normalized to 100% relative abundance. This is followed by the second most abundant molecule, M + 1, which is heavier by 1 mass unit. For instance, the molecules represented by M + 0, M + 1, M + 2 and M + 3 of glucose have a relative natural abundance of 100%, 6.9%, 1.4% and 0.1%, respectively (Kim et al., 2016). This is determined by the relative abundance of carbon-13 isotope and the number of carbons in the molecule.

1.2.1. Expressions of isotopic enrichment for stable isotope kinetics

A metabolic stable isotope tracer is a molecule labelled with one or more stable isotopes, having a different mass from the most abundant form of that particular molecule. In a typical isotope experiment, the organic compound subject to tracing is called the 'tracee' (Chokkathukalam et al., 2014). Once the compound containing the isotopes is administered, the isotopes participate in the same metabolic pathway as the unlabeled tracee and the metabolic fate of the tracer can be monitored by calculating the change in tracer to tracee ratio (TTR) with respect to time, known as enrichment. The mass difference of stable isotopes enables detection *via* MS, or if the isotope possesses the nuclear property of spin it can be detected by NMR spectroscopy (Table 1).

As mentioned above, the 1-^{13}C-glucose tracer (M + 1) is heavier by one mass unit from the most abundant form of naturally occurring glucose, ^{12}C-glucose. This mass difference allows us to track the metabolic fate of the label and calculate *in vivo* kinetics of the tracee. However, the naturally occurring heavier molecule ([1-^{13}C]-glucose) cannot be differentiated from the M + 1 tracer that was infused or injected, therefore, the background enrichment is typically subtracted. The TTR is used to express isotopic enrichment, although it can also be expressed as atom percent excess (APE, %) or molar percent excess (MPE,%). The latter two terms reflect the amount of the tracer as a ratio of the sum of the tracee and tracer (Godin and Schierbeek, 2017).

The TTR is calculated using formula (1) and it can be transformed using formulae (2) and (3):

$$TTR = (r_{sa} - r_{bk}) \times (1 - S)^{n} \quad (1)$$

$$APE, \% = TTR/(1 + TTR) \times 100 \quad (2)$$

$$MPE, \% = APE \times n(C_{total})/n(C_{labelled}), \quad (3)$$

Where r_{sa} is the tracer to tracee ratio in the biological sample after tracer administration, r_{bk} is the tracer to tracee ratio in the baseline sample, before tracer administration, S is the skew correction of a specific isotope (usually approximated by the value [1/(1 + An)],