LSD1 pathways in DOHaD. In the immediate response, Sirt1 promotes OXPHOS activity and slow-twitch fiber formation under catabolic action. In the following adaptive response, LSD1 enhances energy storage and fast-twitch fiber formation, possibly under anabolic conditions. This order of events appears to be critical for the perinatal survival strategy against undernutrition. Obviously, other metabolic tissues, such as the liver, pancreas, kidney, adipose, and brain, are also involved. However, such developmental adaptation could be advantageous or disadvantageous depending on later nutrient availability. Further research on the NAD^+–Sirt1 and FAD–LSD1 pathways is required to understand the long-term effects of the nutritional environment on the epigenome and metabolic phenotypes, which are linked to subsequent disease susceptibility.

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Forum

The Whys and Hows of Calculating Total Cellular ATP Production Rate

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Quantifying total cellular ATP production rate has become easier with recent technology and is essential to understanding energy metabolism in cells and tissues. We review fundamental concepts for determining total cellular ATP production rate from measurements of oxygen consumption and acidification rates and discuss their application to answering biological questions.

Cells Leverage Network Properties to Control ATP Production and Consumption

Current technologies for measuring rates associated with ATP production are increasingly accessible. Some instruments, such as the Agilent Seahorse XF Analyzer, simultaneously measure extracellular acidification and oxygen consumption, which together can be used to calculate total cellular ATP production rate [1]. Others, such as the Oroboros O2k [2], can be configured to include pH measurements for the same goal. These platforms and methods complement a renewed interest in using bioenergetic analysis to explore mechanisms of carcinogenesis and cancer progression, diabetes [3], cellular differentiation [4], and neurodegeneration [5] among others. Here, we review the calculation of cellular ATP production rate and its applications to these fields.

The task of meeting cellular ATP demand in cells, tissues, and organisms is shared between mitochondrial oxidative phosphorylation and glycolytic ATP production. Basal and maximal rates of cellular ATP production, their partitioning between glycolysis and oxidative phosphorylation, and what meaning can be drawn from these measurements are issues of intense current interest. Calculating ATP production rates can reveal the efficiency of energy transduction, and therefore what a cell or organism needs to survive and function. It can reveal the adaptability of ATP production to stresses, and therefore how energetically sensitive (or resistant) biological targets are to drugs or toxins. Combined with control analysis, it can provide new mechanistic insights into complex diseases [3].

Cellular ATP production is driven by a network of linked reactions that achieve stable steady-state fluxes. The principles that govern glycolytic and oxidative ATP production are the same ones that govern other steady-state networks. At the timescale of most measurements, seconds to minutes, these networks operate by and are explained by the interactions between internal forces (levels) and fluxes (rates) [6]. They have internal control; an imposed

change to one force or flux, such as ATP demand, provokes a rapid response in others, such as respiration rate, to establish a new steady state. Importantly, this internal control does not require external intervention (e.g., by enzyme modification or transcriptional alteration). Such interventions usually operate at longer timescales, minutes to days, and alter the dynamic properties of the network; for example, by changing enzyme activity.

Within this network model, powerful hypothesis testing is possible. In cancer, the 'aerobic glycolysis' described by the Warburg effect is perhaps the most discussed phenotype of cancer metabolism, despite considerable uncertainty about what defines it (increased glucose uptake? increased lactate production? increased how much, and relative to what?) and what it represents (mitochondrial dysfunction? increased catabolic flux? increased anabolic flux?). Perhaps as a result, no consensus model exists, despite numerous proposed mechanisms [7,8]. Recent bioenergetic analyses reveal much greater variety and plasticity of basal ATP production than predicted by the Warburg model [9]. The weaknesses of the Warburg effect as a bioenergetic model, combined with contradictory findings, may explain the clinical failure of cancer treatments that target glycolysis [10] and illustrate how entirely different strategies may be needed.

Cellular differentiation is also associated with changes in respiratory and glycolytic fluxes (e.g. [4]). How much these changes are explained by redistribution of ATP supply, rather than by changes in total ATP production, is unclear but could be easily determined. Associations between redistribution of ATP supply and the acquisition of traits may suggest a causal role [11] but evidence is lacking, in part because most reports do not distinguish between loss of the targeted source of production and overall ATP collapse.

As some of the above examples illustrate, many current bioenergetic models overlook or overly complicate the theoretical simplicity of the underlying network. To explain acute responses, external events are not necessarily needed. A second conceptual problem is that terms used to describe energetic phenotypes make mechanistic assumptions that must be tested but often are not. For example, a cell that makes ATP predominantly by glycolysis might be described as having a 'preference' for glycolytic ATP, implying a hierarchy that may or may not exist and ignoring network homeostasis: if a predominantly glycolytic cell becomes predominantly oxidative in another condition, which pathway does it 'prefer'? This problem worsens when a preference becomes the hypothesized 'driver' of a process. These conceptual problems can be avoided by reframing observations of ATP production rates in terms of network homeostasis. In systems where both glycolysis and oxidative phosphorylation are running, ATP production from both must be determined to understand network behavior.

Calculating Total Cellular ATP Production Rate from Shared Supply Pathways

Rates of glycolysis and oxidative phosphorylation are very crudely represented by the extracellular fluxes of acidification and oxygen consumption, respectively, gathered using the Seahorse, Oroboros, or other system. It is hard to meaningfully interpret total cellular bioenergetics from raw extracellular fluxes [1]. However, simple transformations enable meaningful interpretation. Figure 1 outlines the principles that underlie the calculation of total cellular ATP production rate from extracellular fluxes. First, acidification is linearly scaled. All rates are then corrected to isolate the ATP-producing portion, weighted to ATP production, and finally summed to yield total cellular ATP production rate.

Glycolytic ATP is logically categorized as all ATP made in glycolysis. This is straightforward yet requires discarding of the misconception that glycolytic ATP production is strictly anaerobic, with all pyruvate converted to lactate and generating acid. Instead, ATP made during the formation of pyruvate that is subsequently oxidized during respiration is also glycolytic. So, calculating glycolytic ATP requires using the measured rates of both acidification and respiration. The acidification rate is scaled from logarithmic pH change to linear proton production rate, then corrected by subtracting proton production generated from respiratory CO_2, which forms carbonic acid. These steps yield the glycolytic proton production rate, which is then weighted by the amount of ATP produced per lactate-associated proton to yield the rate of lactate-associated glycolytic ATP production. The remaining glycolytic ATP is similarly determined using the respiration rate: the oxygen consumption rate is corrected by subtracting the non-mitochondrial contribution, then weighted by the amount of glycolytic ATP produced per oxygen consumed during subsequent oxidation of the pyruvate. These two rates sum to glycolytic ATP production.

Determining oxidative ATP production is similar. Measured oxygen consumption is corrected to isolate mitochondrial respiration rate. Two different weightings are applied, each to a different portion of respiration; ATP generated by substrate-level phosphorylation in the tricarboxylic acid (TCA) cycle is proportional to all mitochondrial respiration, whereas ATP generated by oxidative phosphorylation is proportional only to coupled respiration. These two rates sum to oxidative ATP production. Finally, all four rates (two in glycolysis, two in respiration) sum to total cellular ATP production.

This calculation can be applied to any system if valid coefficients are used (e.g., for the P/O ratio, which describes how many ATP are made per oxygen consumed). Proper accounting of ATP production, especially where non-glucose substrates are used or where cells are partially oxidizing substrates, requires that P/O values be empirically determined.

Linking Calculation to Application: The Crabtree Effect

As an example of the network model of ATP production and its application to hypothesis testing, consider the Crabtree effect, a long-observed phenomenon in which addition of a sugar to a glycolysis-competent system increases the glycolytic rate and decreases the respiration rate. As a network response (Figure 2), the additional ATP supplied by glycolysis when sugar is introduced meets part of the ATP demand that was previously satisfied by respiration. The ATP/ADP ratio rises as ADP levels fall, causing demand for respiratory ATP to decrease, and respiration therefore slows by a simple kinetic response to high ATP/ADP. Everyday experiences of this phenomenon include the slowing of traffic due to merging lanes.

Modeling the Crabtree effect as a network response explains it entirely, eliminates the need for additional regulation, and illustrates its bioenergetic requirements. A 'Crabtree-competent' cell has demand-driven ATP production and unused glycolytic capacity. The magnitude of the Crabtree response will vary with conditions depending on how strongly glycolytic ATP production is stimulated by substrate addition and whether total ATP demand changes when sugar is added.

By contrast, models that interpret the Crabtree effect as direct respiratory inhibition (rather than a simple kinetic response to increased ATP/ADP) must include inhibitors and inhibited elements. This leads to proposals of metabolite inhibition of a downstream element (e.g., the mitochondrial pyruvate carrier or electron transport complexes [12]). Such proposals can be tested by measuring the strength of product inhibition by ATP/ADP: if it is sufficient to explain the observed Crabtree rate changes, direct respiratory inhibition

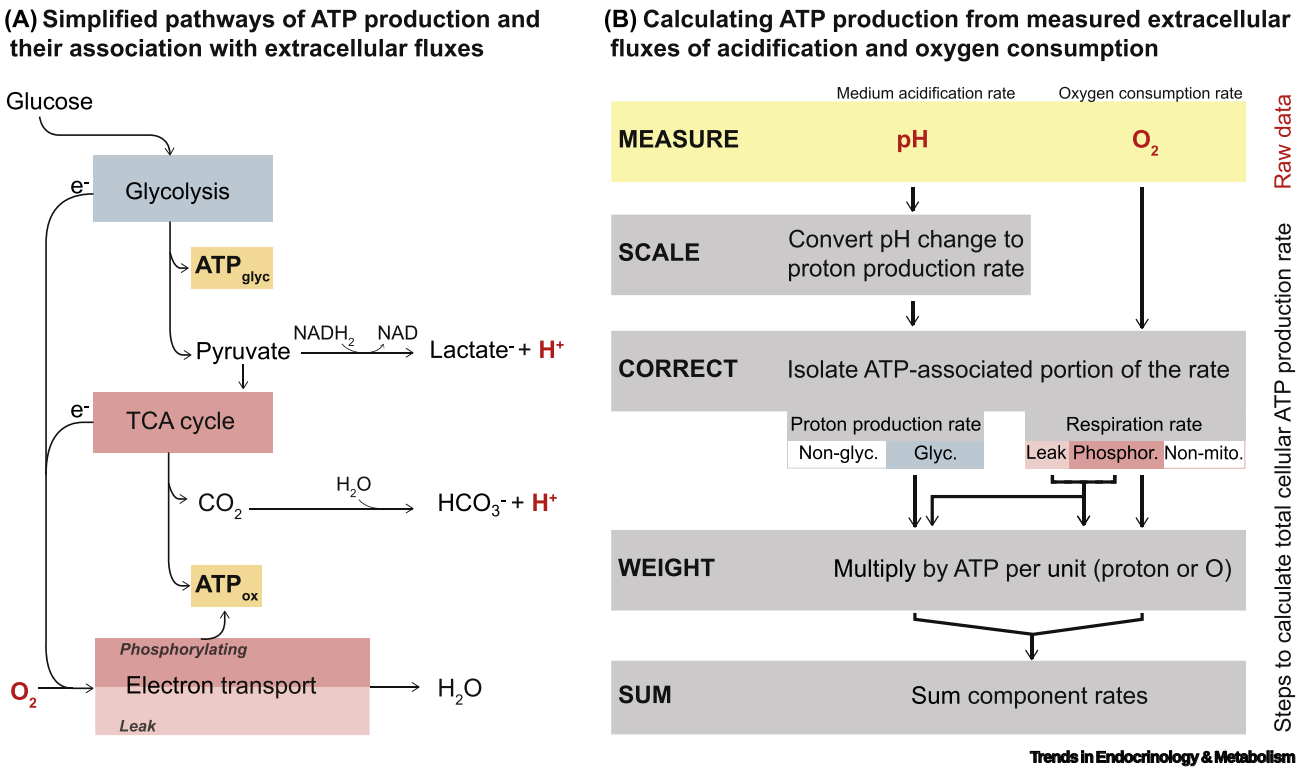

Figure 1. Cellular ATP Production and Its Association with Extracellular Flux Measurements. (A) Glucose is catabolized to lactic acid, CO_2, and H_2O. Glycolysis yields electrons (e^-), glycolytic ATP (ATP_{glyc}), and pyruvate (strictly, pyruvic acid). Pyruvate reduction yields lactate$^-$ and H^+. Tricarboxylic acid (TCA) cycle flux yields e^-, substrate-level oxidative ATP (part of ATP_{ox}), and CO_2. CO_2 hydration yields HCO_3^- and H^+. Electron transport flux drives oxidative phosphorylation to yield the rest of ATP_{ox} and consumes O_2 to yield H_2O. (B) Conversion of extracellular flux measurements to ATP production rates. Glyc., glycolytic; Phosphor., phosphorylating; Mito., mitochondrial. For additional calculation details, see [1].

is not part of the Crabtree mechanism. Of course, the Crabtree effect can be mimicked by direct inhibition of respiration, but this alters the dynamic properties of the network (akin to halting the merging traffic) and does not show how the uninhibited system responds.

Additional Technical Considerations

Any measurement system that captures simultaneous and absolute values of oxygen consumption (change in O_2/time) and acidification (change in pH/time) can provide the data for calculation of ATP production rates using the principles discussed above. The XF and Oroboros systems generate appropriate data, but plate-based fluorescence probes such as Agilent's MitoXpress-Xtra plus pH-Xtra do not; the fluorescence signals would need to be calibrated to allow calculation of total cellular ATP production rate.

As with any biological question, respiratory and glycolytic properties are a function of a cell's growth and assay environments. Experiments must be designed with assay conditions (e.g., oxygen tension, carbon substrates, hormone levels, cell–cell interactions) appropriate for the question being posed. Extrapolation to physiology *in vivo* may be valid but must be tested. Generally, reductionist experimental design, such as cell culture, allows greater experimental control at the cost of physiological relevance. Conversely, physiological systems may be highly relevant but limited in the degree of experimental manipulation possible.

Concluding Remarks

Acute regulation of ATP production is primarily a function of network responses. Understanding these responses requires the determination of total cellular ATP production rates. This can be done by simple calculations based on readily measurable extracellular changes in O_2 and pH, as outlined here. Combining these fundamental concepts with technology and interest can drive significant progress in bioenergetic analysis.

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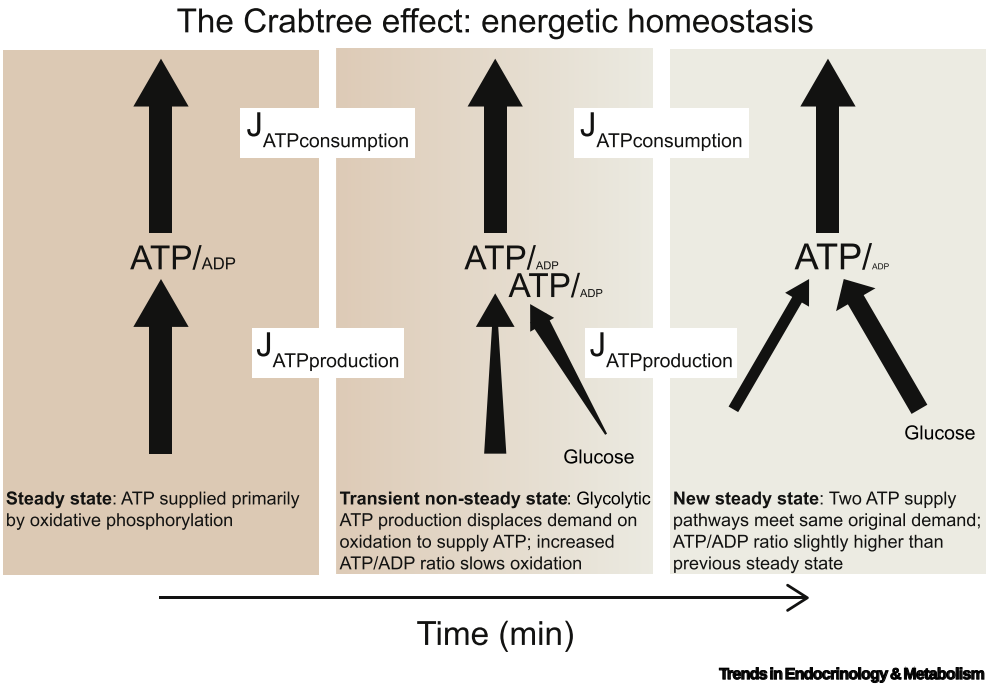

Figure 2. The Crabtree Effect as a Network Response of Energy Homeostasis. Left: Steady-state ATP production in cells lacking sugar occurs primarily through oxidative phosphorylation. Middle: Introducing a sugar (e.g., glucose) drives glycolytic ATP production, increasing ATP/ADP and inducing a transient non-steady state. Product inhibition by ATP/ADP slows oxidative phosphorylation. Right: New steady-state ATP production by two supply pathways. In this example, glycolytic ATP production predominates (thicker arrow) but this proportionality will vary with cell type and assay conditions. Although the Crabtree effect may have different magnitudes *in vivo* and under particular experimental conditions in cultured cells, the network properties that give rise to the Crabtree effect experimentally are likely to be intact *in vivo*.

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REVIEWS

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Spotlight

Adipose Progenitor Cells Contribute to Lipid Spillover during Obesity

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A recent study (Girousse *et al.* *Cell Rep.* 2019;27:323–333) shows that CXCR4$^+$ adipose progenitors (APCs) contribute to lipid spillover during high-fat feeding through their release from subcutaneous fat depots (ScATs) and migration to skeletal muscle where they differentiate into adipocytes. Pharmacological antagonism of CXCR4, which prevents the CXCL12-dependent retention of APCs in ScAT, mimics the effects of overfeeding.

Obesity is the consequence of an imbalance between energy intake and energy expenditure. During the initial stages of obesity, subcutaneous adipose depots (ScATs) expand to accommodate the storage of excess dietary fat. Progressively, the expansion limit is reached and the nonstored lipids start spilling over into other tissues [1]. The resulting deposition of fat in visceral adipose depots, skeletal muscle, liver, pancreas, and myocardium has been positively correlated with insulin resistance and an increased risk of developing metabolic disorders including type 2 diabetes (T2D) and cardiovascular disease [2]. It is widely accepted that the spillover increases the circulating levels of lipids, which are subsequently taken up by cells in ectopic tissues.

In their recent work, Girousse *et al.* refined this spillover concept by demonstrating that adipocyte progenitors (APCs) also participate in the redistribution process [3]. The authors revealed that a subpopulation of APCs expressing the C-X-C chemokine receptor type 4 (CXCR4) are released from the ScAT in response to high-fat feeding and give rise to new adipocytes in skeletal muscle. The detachment of CXCR4$^+$ APCs is promoted by a decrease in the levels of the chemokine CXCL12 in the ScAT microenvironment and by increased secretion in skeletal muscle (Figure 1) [3]. The authors observed that the percentage of this APC subpopulation was inversely correlated with ScAT fat-pad weight. No correlation was noted for the visceral depots even though these CXCR4$^+$ APCs are present in both depots, suggesting a depot-specific response to diet. To determine the fate of the circulating APCs, Girousse *et al.* grafted a piece of ScAT from mice expressing a *Cd34–egfp* transgene (that fluorescently marks APCs – CD34 is a surface marker for APCs) to ScAT of a non-GFP recipient mouse. They demonstrated that, after 8 weeks of high-fat diet (HFD) feeding, the recipient GFP$^+$ APCs could be detected by immunohistochemistry in the quadriceps muscle [3]. This result is consistent with detachment of CD34$^+$ APCs from the graft and their relocation to skeletal muscle. The authors were not able to determine whether the ectopic APCs differentiated into adipocytes within the muscle. This required the use of another mouse ('Ad-Cre/Zs1Green') in which adipocytes could be conditionally marked with GFP by treatment with tamoxifen. As before, a piece of ScAT was grafted from the Zs1Green mouse to the ScAT of a non-GFP recipient. The grafted animals were then treated with tamoxifen 8 weeks after a HFD to induce GFP expression in all adipocytes. Zs1Green$^+$ adipocytes were detected in the quadriceps muscle, confirming not only transit of ScAT-derived APCs to muscle but their differentiation into adipocytes [3]. Accumulation of ZsGreen1$^+$ adipocytes within the quadriceps muscle could also be induced by replacing the HFD diet by weekly injections of 5 mg/kg of the CXCR4 antagonist AMD3100 for 8 weeks. The authors had previously demonstrated that AMD3100 triggered the release of APCs from ScAT [4]. Further studies showed that mice fed a normal chow diet (ND) and 8 weeks of AMD3100 treatment gained weight and increased total fat mass to the same extent as mice fed a HFD. The mass of the ScAT and perigonadal adipose tissue