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The Warburg effect: a balance of flux analysis

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Abstract

Cancer metabolism is characterized by increased macromolecular syntheses through coordinated increases in energy and substrate metabolism. The observation that cancer cells produce lactate in an environment of oxygen sufficiency (aerobic glycolysis) is a central theme of cancer metabolism known as the Warburg effect. Aerobic glycolysis in cancer metabolism is accompanied by increased pentose cycle and anaplerotic activities producing energy and substrates for macromolecular synthesis. How these processes are coordinated is poorly understood. Recent advances have focused on molecular regulation of cancer metabolism by oncogenes and tumor suppressor genes which regulate numerous enzymatic steps of central glucose metabolism. In the past decade, new insights in cancer metabolism have emerged through the application of stable isotopes particularly from ¹³C carbon tracing. Such studies have provided new evidence for system-wide changes in cancer metabolism in response to chemotherapy. Interestingly, experiments using metabolic inhibitors on individual biochemical pathways all demonstrate similar system-wide effects on cancer metabolism as in targeted therapies.

Since biochemical reactions in the Warburg effect place competing demands on available precursors, high energy phosphates and reducing equivalents, the cancer metabolic system must fulfill the condition of balance of flux (homeostasis). In this review, the functions of the pentose cycle and of the tricarboxylic acid (TCA) cycle in cancer metabolism are analyzed from the balance of flux point of view. Anticancer treatments that target molecular signaling pathways or inhibit metabolism alter the invasive or proliferative behavior of the cancer cells by their effects on the balance of flux (homeostasis) of the cancer metabolic phenotype.

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Introduction

The uncontrolled proliferation of cancer cells of various tissue types is still a leading cause of morbidity and mortality in the world. A well known characteristic that separates cancer cells from normal differentiated cells is their increased de novo lipid synthesis, elevated anabolic processes, decreased/altered TCA cycle metabolism, high glucose usage glucose as an energy and carbon source for cell growth and division. This unusual utilization of glucose results in the production of lactate in the presence of oxygen sufficiency (aerobic glycolysis) and is known as the Warburg effect (Upadhyay et al., 2013, Ferreira, 2010). Recent advances in molecular biology have identified oncogenes and tumor suppressor genes that play a role in the regulation of metabolism through their regulation of signaling pathways and enzyme expression (Faubert et al., 2013, Feng and Levine, 2010, Levine and Puzio-Kuter, 2010, Zheng, 2012). Oncogenes such as myc and ras, and suppressor genes such as p53 are transcriptional factors or signaling molecules that are normally present in cells. Oncogenes and tumor suppressor genes regulate metabolic enzyme expression, activities and ultimately the metabolic phenotype. A mutation of one of these genes predisposes the cell to the development of a cancer or the Warburg phenotype (Vizan et al., 2005). However, experiments using pathway specific metabolic inhibitors all demonstrate system-wide effects on cellular metabolism. For example, inhibition of glycogenolysis by glycogen phosphorylase inhibitor (CP-320626) resulted in significant reduction in both oxidative and non-oxidative pentose cycle activities, and decreased pyruvate dehydrogenase activity and de novo lipogenesis in pancreatic cancer MIA PaCa-2 cells apoptosis (Lee et al., 2004). Similar metabolic changes in pentose cycle and de novo lipogenesis were observed when lactic acid production was inhibited by oxamate, a lactic acid dehydrogenase (LDHA) inhibitor (Lu et al., 2014). Inhibition of signaling pathways also can impact on cancer cell metabolism and growth. Interestingly, inhibition of Bcr-Abl tyrosine kinase with imatinib mesylate (STI571) in K562 human leukemia cells affects hexokinase, G6PDH and transketolase metabolic pathways which are dominant in the Warburg effect (Boren et al., 2001). The fact that targeting signaling or metabolic pathways of a metabolic system can arrive at similar metabolic endpoints readily suggests a certain metabolic organization and a common ground rules in systems biology, which is the physical and chemical constraints¹, and the balance of flux. The concept of balance of flux is applicable to reactions in one functional or physical compartment, as well as in all the compartments of the cellular metabolic network as a whole. In this review several components of the Warburg effect are discussed as two separate major compartments the cytosolic and the mitochondrial compartments.

Balance of flux in systems biology

Balance of flux is a basic concept in chemistry. In a basic chemical equation, changes in mass, charge and energy on one side of the equation must equal changes to those on the

¹In a cellular system, biochemical reactions are constrained by physical and chemical factors such as compartmentalization or tunneling; K_m and V_{max} of Michaelis Menten kinetics; cofactor concentrations, and conservation of mass and energy (balance of flux).

other side of the equation. It is an expression of conservation of matter and energy. A metabolic network is consisted of a large number of biochemical reactions. These reactions are of two major types²:

$$\text{Substrate input} = \text{metabolic intermediates} + \text{energy} + \text{byproducts} \quad (\text{Equation 1})$$

$$\text{Metabolic intermediates} + \text{energy} = \text{metabolic products} + \text{byproducts} \quad (\text{Equation 2})$$

Energy output in both equations is in the form of high energy phosphate bonds and reducing equivalents. The substrates and metabolic intermediates are mostly interconvertible going from a reduced state to an oxidized state and *vice versa*. In order for the system to function efficiently, these two sets of equations must be satisfied simultaneously. The idea of balance of flux has been applied to *in-silico* modeling of bacterial metabolism (Reed and Palsson, 2003). Equations of flux balance are first expressed in terms of stoichiometries of individual substrates, and in the form of a matrix, the stoichiometric matrix. Since there are more unknowns than equations, the mathematical technique linear programming is employed to solve the set of balance of flux equations for the entire network. The result is an n-dimensional convex conical solution space. The convex cone can be resolved into a series of two dimensional phenotypic phase planes representing the metabolic phenotype of the metabolic network (Edwards et al., 2002, Lee and Go, 2005, Orth et al., 2010, Paul Lee et al., 2010, Schilling et al., 2000, Schilling and Palsson, 1998). The concept of balance of flux is scalable³. It is applicable to a system of biochemical reactions in a cell as well as in a group of cells in a tissue. The biochemical reactions of the Warburg effect are part of the cellular metabolic network. Each series of reactions places competing demands on available precursors, high energy phosphates and reducing equivalents. In order for these biochemical reactions to occur simultaneously, they must fulfill the condition of balance of flux (homeostasis). Therefore, alteration in any part of the system potentially affects the balance of flux in the rest of the system.

In a recent study of the effect of oxamate on the Warburg phenotype (Lu et al., 2014), it was observed that inhibition of LDHA had upstream and downstream metabolic consequences suggesting the possibility of three functional compartments, namely i) glycolytic/pentose cycle, ii) lactate/pyruvate and iii) mitochondrial/TCA compartments. These three functional compartments within the metabolic system are also suggested by the observations in the “glucose paradox” (Katz and McGarry, 1984). Glucose is well known as the major substrate for hepatic metabolism after insulin stimulation. Paradoxically, glucose is a poor substrate for liver glycogen and triglycerides synthesis as compared to lactate, fructose or glutamine. This is because lactate, fructose and glutamine are metabolic intermediates in their respective functional compartments. The supply of these intermediates alters the flux balance in each respective compartment and increases glycogen and fatty acid synthesis

²The two types of reactions of equation 1 and 2 are either energy consuming or energy producing reaction. There is a large group of reversible reactions in which energy state of the system is not affected.

³The concept of balance of flux is applicable to reactions in one functional or physical compartment, as well as in all the compartments of the cellular metabolic network as a whole.

mimicking the effect of insulin (Marin et al., 2004). The distinctive features of these compartments with respect to the balance of flux are presented below.

The Glycolytic/Pentose cycle compartment

The central theme of Warburg effect is aerobic glycolysis. Glucose is metabolized into lactate through an inefficient energy producing pathway (Figure 1) despite of the increased energy requirement for growth and proliferation. However, the glycolytic pathway is not the only pathway that can convert glucose into lactate (Fig. 1). Figure 2A shows the oxidative branch of the pentose phosphate pathway (PPP) and the biochemical reactions are summarized in Figure 1.

These two pathways are virtually indistinguishable when one just measures glucose uptake and lactate production as commonly done. However, the potential for energy production is much greater through PPP than through glycolysis. When triose phosphate is converted back to glucose-6-phosphate (G6P) by the transaldolase/transketolase pathways, the oxidative branch of the pentose cycle can continue with additional glucose input, and the net result is equivalent to that of one glucose converted to six carbon dioxides and 12 reducing equivalents. In reality, glycolysis and the pentose cycle do not operate independently. The traditional view of energy production in Warburg effect grossly underestimates the potential energy production in this compartment.

The intermingling of metabolic intermediates of glycolytic and the pentose pathways and the sharing of intermediates have been demonstrated using stable isotope tracers. These pathways share common substrates such as G6P, fructose-6-phosphate (F6P) and triose-P; and constraints such as the availability of ATP, ADP and NAD. Previously, when HepG2 cells were cultured in the presence of [1, 2 $^{13}\text{C}_2$]-glucose, four major isotopomers were found in RNA ribose. These are [1- ^{13}C]-, [5- ^{13}C]-, [1, 2 $^{13}\text{C}_2$]- and [4, 5- $^{13}\text{C}_2$]-ribose. The singly-labeled ribose is derived from the oxidative branch of the pentose cycle while the doubly-labeled ribose is derived from the non-oxidative branch. The fraction of [1- ^{13}C]-ribose is about ten times that of the [5- ^{13}C]-ribose which is in agreement with Figure 1A in that [1- ^{13}C]-ribose is unlikely to be recycled to form [5- ^{13}C]-ribose. The fractions of [1, 2 $^{13}\text{C}_2$]- and [4, 5- $^{13}\text{C}_2$]-ribose were about equally distributed because of randomization of triose-P by isomerase (Lee et al., 1998). The labeling patterns in glucose and ribose due to the actions of glycolytic and pentose pathways have also been demonstrated using [U- $^{13}\text{C}_3$]-glycerol in serum starved FAO cells (Kurland et al., 2000). Stable isotope labeling experiments not only can provide specific information regarding pathways but also be used to estimate metabolic flux. Previously, ribose isotopomer distribution from [1, 2 $^{13}\text{C}_2$]-glucose has been used to estimate PPP flux using computer modeling (Selivanov et al., 2005, Selivanov et al., 2004). The non-oxidative branch of the PPP is shown in Figure 2B. For the same concentrations of PPP intermediates, five molecules of glucose can be converted to six pentose phosphates at the expense of six molecules of ATP.

The Glycolytic/Pentose cycle compartment is responsible for the production of energy rich compounds and ribose and deoxyribose for nucleoside synthesis. In order to balance glucose utilization with production of energy rich compounds, lactate and sugar phosphates, the

reactions listed in Figure 1 have to be satisfied simultaneously as in constraint-based modeling (Orth et al., 2010). Figure 2 illustrates an important concept in metabolism at steady state. As shown in this figure, glucose can be converted to its products without changing the concentrations of the intermediates (fructose-6-P, sedoheptulose-7-P and erythrose-4-P) (Fig. 2), and changes in the concentrations of any one of the intermediates can increase or decrease the rate and output of the pentose cycle

In addition to substrate and co-factor constraints, metabolic activities in this compartment are dependent on substrate input regulated by glucose uptake and phosphorylation by hexose kinase into glucose-6-phosphate (G6P). G6P is used in three different reactions: glycogen synthesis, glucose-6-phosphate dehydrogenase (G6PDH) for the PPP; and fructose-1,6-bis-phosphate (F-1,6-bis-P) by phosphofructose kinase (PFK1) for glycolysis. Glycogen serves as an overflow compartment, maintaining a steady state G6P concentration. The conversion of F-1,6-bis-P to pyruvate by phosphoglycerate mutase (PGM) regulates the rate of glycolysis (Levine and Puzio-Kuter, 2010). (Supplemental Figure 1S) These regulatory reactions can be activated by the tyrosine kinase receptor and its downstream signaling pathways. Many of the enzymes in this glycolysis/pentose cycle compartment are inducible by the hypoxia induced factor 1 (HIF-1) (Kim, Gardner and Dang, 2005). The metabolic activities and enzyme levels are further modulated by the action of p53 (tumor suppressor gene) forming a normal feedback loop in normal cells. The Warburg phenotype is observed when this feedback mechanism is lost allowing uncontrolled growth and proliferation (Levine and Puzio-Kuter, 2010, Feng and Levine, 2010).

The Mitochondria/TCA cycle compartment

Pyruvate is the key metabolite that links glycolysis and pentose cycles with mitochondria metabolism. Pyruvate is produced mainly from triose-P by pyruvate kinase (PKM2) and is in equilibrium with lactate by the reversible action of lactic acid dehydrogenase (LDHA) and alanine by transaminase. It is the major source of oxaloacetate and acetyl-CoA. The action of pyruvate dehydrogenase (PDH) results in irreversible loss of 3-carbon compounds such as pyruvate (Figure 3). The partitioning of pyruvate between pyruvate carboxylase and pyruvate dehydrogenase pathways has been demonstrated using ^{13}C glucose or lactate by the relative positional mass isotopomers in α -ketoglutarate (α -KG) or glutamate. The condensation of oxaloacetate (OAA) and acetyl-CoA forms specific position and mass isotopomers in citrate and α -KG. ^{13}C -OAA introduces ^{13}C -labels to carbons 1 to 3 of α -KG, while acetyl-CoA labels carbon position 4 and 5 of α -KG. The distribution of mass and position isotopomers in glutamate therefore can be used to determine the relative flux between these two pyruvate reactions (Lee, 1993, Katz et al., 1993, Katz et al., 1989). Production of acetyl-CoA for de novo lipogenesis and formation of oxaloacetate in anaplerosis are two major uses of pyruvate. Since acetyl-CoA can be used either for TCA cycle or de novo lipogenesis, in proliferating cells such as cancer glucose is generally not used primarily for the production of 36 ATP by OXPHOS in cellular metabolism⁴.

⁴Glucose is the ultimate source of energy and carbon for amino acids and fatty acid synthesis. As a general rule in metabolism, energy is conserved (stored) in the form of fatty acids, and carbon source is conserved or regenerated through anaplerosis. Thus, glucose is generally not used for ATP production, which is consistent with the occurrence of hypoglycemia in fatty acid oxidation abnormality and the development of diabetes under high fat dietary condition.

Beta-oxidation and the TCA cycle begin with the condensation between oxaloacetate (OAA) and acetyl-CoA forming citrate. The sequence of reactions and the major intermediates α -ketoglutarate (α -KG), succinyl-CoA, fumarate and malate are shown in Figure 3. Equations for the biochemical reactions of the TCA cycle are shown in Figure 4. The reactions that are tangential to the TCA cycle are shown in Supplemental Figure S2. TCA cycle flux depends on the steady state concentration of its intermediates. The rate of TCA cycle flux can be increased or decreased by the increase or decrease the levels of the intermediates (citrate (CIT), α -KG, malate and OAA). The rate of oxidative phosphorylation (OXPHOS) in cells parallels the production of the reducing equivalents from TCA cycle flux.

The filling of TCA cycle intermediate pools is known as anaplerosis and reactions that fill the TCA cycle intermediate (OAA, CIT, α -KG, and Malate) are known as anaplerotic reactions (Figure 5). The balance of flux equations of these reactions are shown in Supplemental Figure S2. Pyruvate carboxylation is the primary source of OAA and other TCA cycle intermediates at steady state. Thus an increase in OAA concentration is often accompanied by a parallel increase in TCA cycle flux and OXPHOS. This association does not hold when TCA cycle intermediates are depleted by the anaplerotic reactions (Figure 5). OAA can be removed from the TCA cycle by the formation of phosphoenolpyruvate (PEP) by phosphoenolpyruvate kinase (PEPCK) or by transamination into aspartate. The formation of PEP is part of the gluconeogenic pathways using ATP and reducing equivalents from the oxidation of acetyl-CoA. Citrate can be removed from the TCA cycle initiating de novo lipogenesis by the export of citrate and its subsequent dissociation by citrate lyase. This series of reactions in de novo lipogenesis has the effect of reducing TCA cycle intermediate concentrations. The reaction by isocitrate dehydrogenase (IDH) produces CO_2 and α -KG. The reverse reaction of IDH in forming isocitrate and citrate from α -KG is part of a futile cycle⁵ that allows the use of glutamate or α -KG for fatty acid synthesis, and has the effect of slowing down TCA cycle flux. The conversion of α -KG to succinyl-CoA by α -ketoglutarate dehydrogenase is an irreversible reaction among the TCA cycle reactions. The interconversion of succinyl-CoA and succinate is another futile cycle. Since α -KG is a precursor for a large number of metabolites such as proline, glutamate, ornithine, and purine and pyrimidine, the demand for substrates is balanced by glutamate uptake and contribution from IDH. Therefore the uptake of glutamate does not necessarily imply increased TCA cycle flux or OXPHOS. To complete the TCA cycle, succinate is converted to malate by succinate dehydrogenase and fumarate hydratase (fumarase). Malate can be exported to the cytosolic compartment, which also acts as a stop for the TCA cycle flux. In ordinary circumstances, NAD^+ and FAD must be regenerated by electron chain transport (ETC) and OXPHOS to allow for continuous operation of the TCA cycle, and the rate of OXPHOS depends on the availability of reducing equivalent generated by TCA cycle flux. It should be pointed out that OXPHOS is not an efficient process that only 33% of available energy is transferred from NADH to ATP. The remainder of the energy from NADH is accounted for by reactive oxygen species and heat production.

⁵Futile cycle refers to a pair of reversible reactions in which the net result is the consumption of ATP or NADPH. A typical example of a pair of reactions is the combined action of glucokinase and glucose-6-phosphatase. It should be noted that these reactions do not take place contemporaneously, and reaction in either direction potentially affects its "downstream" reactions.

Anaplerosis which fills and removes TCA cycle intermediates from the mitochondria is one of the regulatory mechanisms of TCA cycle and OXPHOS. Anaplerosis relative to TCA cycle flux is an important measure of mitochondrial function and has been measured using ^{13}C stable isotope labeled glucose, lactate or acetate (Lee, 1993, Katz et al., 1989, Vaitheesvaran et al., 2014). The measurement of anaplerotic flux depends on the quantitation of mass and position isotopomer in glutamate as surrogate for isotopomer in α -KG. When $[1, 2\text{-}^{13}\text{C}_2]$ -glucose is used as the tracer, pyruvate carboxylase introduces ^{13}C to carbon positions 3 and 4 of glutamate which becomes $[1, 2\text{-}^{13}\text{C}_2]$ - or $[3, 4\text{-}^{13}\text{C}_2]$ -malate. The trifluoro-acetamide butyl ester (TAB) derivative of glutamate is analyzed by gas chromatography/mass spectrometry (GC/MS) yielding a fragment that corresponds to carbons 2–5 of glutamate. (Please also see isotopomer figures in references below.) In this fragment, the initial labeling of carbons 2–3 gives a m2 ($2\text{-}^{13}\text{C}$'s) isotopomer and the ^{13}C in the second turn of the TCA cycle becomes m1 (singly labeled) in positions of carbon 2 or carbon 3⁶. The ratio of m1/m2 can be used to calculate anaplerotic flux relative to that of the TCA cycle (Lee, 1993, Lee et al., 1996)⁷.

The oxidation of acetyl-CoA through the TCA cycle is modulated by anaplerotic reactions shown in Fig. 5. It can also be altered by hereditary or somatic mutation of the TCA cycle enzymes giving rise to a truncated TCA cycle (Chen et al., 2013, Turcan et al., 2013, Chaturvedi et al., 2013). Succinate dehydrogenase (SDH) and fumarate hydratase (FH) are two enzymes the sequential actions of which convert α -KG to malate. Mutations of these enzymes are associated with familial paraganglioma, and leiomyoma and papillary renal cell cancer, respectively. These enzyme abnormalities impair TCA cycle flux. In a recent study by Yang et al., utilization of glucose by FH null cells was compared with that of cells with FH activity restored (FH/WT) (Yang et al., 2013). Oxygen consumption by FH null cells was only one quarter of the FH/WT cells, incorporation of ^{13}C from $[1, 2\text{-}^{13}\text{C}_2]$ -glucose into glutamate and aspartate was virtually absent in the FH cells. Such observations are consistent with our understanding that TCA cycle flux generates reducing equivalents which drive oxygen consumption and OXPHOS. Another consequence of truncated TCA cycle flux is that the remaining OXPHOS is supported by the influx of α -KG from glutamate. The excess α -KG can be used to produce acetyl-CoA from citrate reductive carboxylation and ATP-citrate lyase. Another tumor associated TCA cycle enzyme abnormality that has generated much interest in targeting cancer energy metabolism as a therapeutic approach is isocitrate dehydrogenase IDH1 mutation. IDH is an enzyme that catalyzes the reversible reaction converting isocitrate to α -KG. IDH1 mutation is known to favor reductive carboxylation and the production of 2-OH-glutarate (the reduced product of α -KG). In an effort to delineate the role of IDH1 mutation in cancer metabolism, IDH1 mutation was introduced to a colorectal carcinoma cell line (Reitman et al., 2014). Metabolic pathways were traced using $[1, 2\text{-}^{13}\text{C}_2]$ -glucose and $[\text{U}^{13}\text{C}_5]$ -glutamine. Increased de novo lipogenesis was found in both IDH1 knock-in cells and cells treated just with 2-OH-glutarate (2HG). Functionally truncated TCA cycle was compensated by increased glucose flux into the TCA cycle intermediates. However, ribose synthesis was impaired in the IDH1 knock-in cells but

⁶ $[1, 2\text{-}^{13}\text{C}_2]$ -OAA is converted to $[1, 2\text{-}^{13}\text{C}_2]$ -glutamate and $[3, 4\text{-}^{13}\text{C}_2]$ -OAA to $[3\text{-}^{13}\text{C}]$ -glutamate. The carbon 2–5 fragment has only singly labeled mass isotopomer from recycling.

⁷The relation between m1/m2 ratio (r) and relative anaplerotic flux (Y) is given by the equation: $Y = (1-r)/r$.

not the 2HG treated cells. These two ^{13}C -carbon tracing studies of truncated TCA cycle (FH and IDH mutations) have not clarified the role of OXPHOS in cancer energy metabolism. However, tracer evidence in these studies suggests a potential role of anaplerotic reactions for substrate production.

Discussion and closing remarks

The original observation by Otto Warburg that cancer cells metabolize glucose via glycolysis, a less efficient pathway for generating ATP despite oxygen availability, appears to be a paradoxical phenomenon which has not been satisfactorily resolved (Levine and Puzio-Kuter, 2010, Ferreira, 2010). The difficulty in resolving this paradox lies in the hidden assumptions of the Warburg effect. It is common assumed that lactate is solely produced by glycolysis and that ATP production by OXPHOS is the most efficient way of producing useful energy. The second assumption is that cancer cells depend on mitochondria metabolism to produce ATP and glucose carbon is primarily used for ATP production. Current investigative techniques into the regulation of cancer cell metabolism by signaling pathways and transcriptional factors are incapable of validating these assumptions. Whether these assumptions are materially correct requires quantitative data such as the distribution of glucose carbons among its metabolic intermediates and how much glucose is used for NADH or ATP production. The use of ^{13}C tracers and mass isotopomer analysis is currently the preferred technique in generating quantitative data (isotopologue) (Boros et al., 2002a, Boren et al., 2003) and such data can be interpreted using balance of flux analysis using tracer-based metabolomics and phenotypic phase plane analysis (Orth et al., 2010, Lee, 2006). For example, we have shown using balance of flux analysis that the oxidative branch of the PPP can produce a similar amount of lactate and generate more reducing equivalents than glycolysis.

The present review of some of the isotopomer data regarding the pentose cycle and the TCA cycle supports the idea of two separate energy producing systems, the glycolytic/pentose cycle component and the mitochondrial component, one producing reducing equivalents (NADH) and the other high energy phosphate bonds (ATP). Reducing equivalents are best used for anabolic processes such as fatty acid synthesis and nucleic acid pentose synthesis, while ATP's are used for macromolecular synthesis, substrate transports and generation of electrical and mechanical energy. These systems are linked by their shared substrates and co-factors, and cellular energy metabolism works like a hybrid engine transforming chemical energy in the cytosolic compartment by the pentose pathway and in the mitochondria by TCA cycle and OXPHOS. It should be reminded that converting reducing equivalent to ATP is an inefficient energy transfer process that generates reactive oxygen species as one of the byproducts, and results in irreversible loss of gluconeogenic substrates.

For any given microenvironment and genotypic endowment of a cell, this hybrid engine has a wide dynamic range basically covering the whole spectrum of metabolic phenotype of cells (Figure 6). Stem cells and cancer cells have a high anabolic requirement. In these cells, PPP can be activated by growth signaling pathways such as $\text{TGF}\beta$ (Boros et al., 2000). Since thiamine is a cofactor for transketolase/transaldolase enzyme, thiamine deficiency either because of genetic transport defect as in thiamine response megaloblastic anemia (TRMA)

(Boros et al., 2003) or anti-thiamine treatment with oxythiamine, retards cell growth which can be rescued with thiamine supplementation (Rais et al., 1999, Comin-Anduix et al., 2001). On the other hand, cardiac myocytes represent the opposite end of this metabolic phenotype spectrum being obligatory ATP producers. In cardiac myocytes, TCA cycle flux is driven by the oxidation of acetate from fatty acids and thus glutamate is heavily labeled in the carbon 4 and 5 positions from ^{13}C -palmitate and not from glucose (Malloy et al., 1990). Living cells are capable of using PPP or OXPHOS depending on the kind of energy need and in response to hormonal influences. Hepatocytes can switch from anabolic to catabolic states in response to feeding and fasting. This bimodal function can be seen from the change in respiratory quotient from fed to fasted states (Xu et al., 2006). Since cells from different tissues all have the same genetic make-up, how these cells acquire their respective metabolic phenotypes is poorly understood. We have previously observed that oxygen induced differentiation in fetal lung lipofibroblast cells from rat pups at two postnatal ages. High oxygen exposure (95%) *in vitro* induced transdifferentiation and metabolic phenotype changes more in cells isolated from younger than older pups (Boros et al., 2002c). Similar proliferative metabolic phenotype changes were observed in fetal rat lung alveolar type II cells after *in utero* nicotine exposure (Rehan et al., 2007). These studies suggest metabolic phenotypes can be acquired during differentiation in cells with proliferative potential such as stem cells or progenitor cells under environmental pressure (Boros et al., 2002b, Lee and Go, 2005). How these genetic and environmental factors contribute to the development of metabolic phenotype is an important question for the understanding of growth and differentiation, and oncogenesis, remaining largely unanswered.

In vitro experiments using metabolic inhibitors on individual biochemical pathways or agents targeting oncogenic pathways all demonstrate similar system-wide effects on cancer cell metabolism (Boros et al., 2001, Harris et al., 2012, Lee et al., 2004, Lu et al., 2014, Boren et al., 2001). The major therapeutic effects on cancer metabolic phenotypes consist of reduced pentose cycle flux in the cytosol and reduced anaplerosis in the mitochondria that result in limiting tumor growth and eventually apoptosis. These effects are also reflected in changes in cell signaling in the cell cycle and apoptotic pathways (Zhang et al., 2010, Ma et al., 2012, Wang et al., 2013). In drug development, the effectiveness of chemotherapy is traditionally measured by the extent of apoptosis by *in vitro* and *in vivo* studies. Since cancer chemotherapies are inherently non-specific especially with anti-metabolites and metabolic inhibitors; normal cells with similar metabolic phenotype are equally affected. When cytotoxic doses are used, common complications result from the development of drug toxicity due to loss of normal cells or the development of drug resistance by environmental selection. In order to avoid such complications, chemotherapy can be tailored for a particular cancer metabolic phenotype using a combination of metabolic inhibitors, the effects of which can be monitored by observing their effects on metabolism. Strategies for such an approach, requires the understanding of the metabolic system and the impact of metabolic inhibitors on the system (Ramos-Montoya et al., 2006). These effects can be demonstrated *in vivo* and *in vitro* using ^{13}C tracers and balance of flux analysis, but such studies are often lacking in the development of cancer chemotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Anaerobic glycolysis

- 1) $\text{Glucose} + 2 \text{ ATP} = 2 \text{ Triose-P} + 2 \text{ ADP}$
- 2) $2 \text{ Triose-P} + 2 \text{ NAD}^+ + 4 \text{ ADP} = 2 \text{ Pyruvate} + 4 \text{ ATP} + 2 \text{ NADH} + 2 \text{ H}^+$
- 3) $2 \text{ Pyruvate} + 2 \text{ NADH} + 2 \text{ H}^+ = 2 \text{ Lactate} + 2 \text{ NAD}^+$
- 4) $\text{Glucose} = 2 \text{ Lactate} + 2 \text{ ATP}$

Pentose cycle

- 1) $3 \text{ Glucose} + 3 \text{ ATP} + 6 \text{ NAD}^+ = 3 \text{ Pentose-P} + 6 \text{ NADH} + 3 \text{ ADP} + 3 \text{ CO}_2 + 6 \text{ H}^+$
- 2) $2 \text{ Pentose-P} = \text{Sedoheptulose-P} + \text{Triose-P}$
- 3) $\text{Sedoheptulose-P} = \text{Erythrose-P} + \text{Triose-P}$
- 4) $\text{Erythrose-P} + \text{Pentose-P} = \text{Fructose-P} + \text{Triose-P}$
- 5) $\text{Fructose-P} + \text{ATP} = 2 \text{ Triose-P} + 2 \text{ ADP}$
- 6) $5 \text{ Triose-P} + 5 \text{ NAD}^+ + 10 \text{ ADP} = 5 \text{ pyruvate} + 10 \text{ ATP} + 5 \text{ NADH} + 5 \text{ H}^+$
- 7) $5 \text{ Pyruvate} + 5 \text{ NADH} + 5 \text{ H}^+ = 5 \text{ Lactate} + 5 \text{ NAD}^+$
- 8) $\text{Glucose} + 2 \text{ NAD} = 1 \frac{2}{3} \text{ Lactate} + \text{CO}_2 + 2 \text{ ATP} + 2 \text{ NADH} + 2 \text{ H}^+$

Figure 1.

Balance of flux equations for anaerobic glycolysis and pentose phosphate cycle showing similar lactate production but substantial difference in energy production of these processes. These equations form the basis of a balance of flux model for the glycolytic/pentose cycle compartment.

Sugar Phosphate System

Figure 2.

Pentose phosphate Pathways (PPP). Fig. 2A shows the PPP operating in the oxidative mode producing NADH and triose phosphates. Fig. 2B shows the PPP operating in the non-oxidative mode producing pentose phosphate. Whether PPP operates in the oxidative or the non-oxidative mode depends on relative contribution of G6PDH and transketolase/transaldolase action, substrate inputs (G6P, F6P, Triose-P (3C)), NADH/NAD ratio and the removal of F6P by the formation of F1,6 diP, and triose-P by pyruvate kinase. The rate of PPP is reflected by the concentrations of the intermediates shown in the figures F6P (6C), Pentose-P (5C), sedoheptulose-P (7C) and erythrose-P (4C). These intermediates are parts of the sugar phosphate system consisting of ketoses, aldoses and sugar alcohols. Oxidation of these compounds initiates their catabolism and conversion to amino acid precursors. Notable example is the oxidation of glycerol to pyruvate and lactate.

TCA Cycle System

Figure 3.

Tricarboxylic acid (TCA) compartment. The pyruvate compartment connects the glycolytic/ pentose cycle compartment to the TCA cycle compartment shown on top of the figure. The reactions in this compartment are shown in Figures 5 and 6. The input into the TCA cycle system starts with the carboxylation of pyruvate to OAA (4C), and condensation of OAA with acetyl-CoA forming citrate (6C). Energy is released in subsequent segments of the TCA cycle. Therefore, interruption of the cycle by the loss of intermediates through anaplerosis can impair the progression of the TCA reducing the rate of oxygen consumption and ATP production. The role of anaplerosis in OXPHOS is also predicted by the balance of flux consideration on equations in Figures 4 and 2S. The TCA cycle intermediates are the precursors of a large number of biosynthesis such as fatty acids and sterols synthesis from acetyl-CoA, and synthesis of compounds such as proline, ornithine, and polyamines from α -ketoglutarate and glutamate.

Tricarboxylic acid cycle

Acetyl-CoA + Oxaloacetate = citrate + CoA-SH

Citrate = Isocitrate

Isocitrate + NAD = α -ketoglutarate + NADH + CO₂

α -ketoglutarate + CoA-SH + NAD = Succinyl-CoA + NADH + CO₂

Succinyl-CoA + GDP = Succinate + GTP

Succinate + FAD = fumarate + FADH

Fumarate + H₂O = Malate

Malate + NAD = Oxaloacetate + NADH

Figure 4.

Balance of flux equations for TCA cycle assuming constant levels of intermediates. Energy from reducing equivalents FADH and NADH are transferred through the electron chain transport (ECT) ultimately reacting with oxygen to generate ATP. Reactive oxygen species and heat are generated as byproducts of OXPHOS.

Anaplerotic Reactions

Figure 5.

Anaplerotic reactions are reactions that can fill or drain intermediates from the TCA cycle. TCA cycle intermediates are shown in text-box and the anaplerotic reactions are indicated by the dotted lines. In the normal operation of the TCA cycle, the primary source of TCA cycle intermediates is from conversion of pyruvate to oxaloacetate (OAA). In cancer metabolism, a large amount of glutamate and aspartate is required for nucleic base synthesis. Input from glutamate and aspartate is often necessary to maintain a high rate of cell proliferation. Conversion of pyruvate to OAA, OAA to PEP and α -KG to succinyl-CoA are the only irreversible reactions. Interconversion of these substrates results in futile cycling and consumption of ATP.

Spectrum of Metabolic Phenotype

Figure 6.

Energy metabolism in cells comprises of a spectrum of anabolic and catabolic activities. These activities are driven by a hybrid system; one generating predominantly NADH and the other ATP. Previous tracer studies have shown cells capable of utilizing these two energy source for cell proliferation and other metabolic functions. Effective cancer treatment mostly affects the anabolic system and anaplerosis. Evidence is lacking as to the effectiveness of inhibiting OXPHOS as a cancer treatment.