

## Pyruvate kinase type M2: a crossroad in the tumor metabolome

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Cell proliferation is a process that consumes large amounts of energy. A reduction in the nutrient supply can lead to cell death by ATP depletion, if cell proliferation is not limited. A key sensor for this regulation is the glycolytic enzyme pyruvate kinase, which determines whether glucose carbons are channelled to synthetic processes or used for glycolytic energy production. In unicellular organisms pyruvate kinase is regulated by ATP, ADP and AMP, by ribose 5-P, the precursor of the nucleic acid synthesis, and by the glycolytic intermediate fructose 1,6-P<sub>2</sub> (FBP), thereby adapting cell proliferation to nutrient supply. The mammalian pyruvate kinase isoenzyme type M2 (M2-PK) displays the same kinetic properties as the pyruvate kinase enzyme from unicellular organisms. The mammalian M2-PK isoenzyme can switch between a less active dimeric form and a highly active tetrameric form which regulates the channeling of glucose carbons either to synthetic processes (dimeric form) or to glycolytic energy production (tetrameric form). Tumor cells are usually characterized by a high amount of the dimeric form leading to a strong accumulation of all glycolytic phosphometabolites above pyruvate kinase. The tetramer-dimer ratio is regulated by ATP, FBP and serine and by direct interactions with different oncoproteins (pp60<sup>v-src</sup>, HPV-16 E7). In solid tumors with sufficient oxygen supply pyruvate is supplied by glutaminolysis. Pyruvate produced in glycolysis and glutaminolysis is used for the synthesis of lactate, glutamate and fatty acids thereby releasing the hydrogen produced in the glycolytic glyceraldehyde 3-phosphate dehydrogenase reaction.

### Immune cells: Nutrients: Serinolysis: Fatty acids

#### Pyruvate kinase, the central nutrient supply sensor in unicellular organisms

Cell proliferation is a process that consumes large amounts of energy. A reduction of the nutrient supply leads to cell death if cell proliferation is not limited (Mazurek *et al.* 1997a, 1999a; Shim *et al.* 1998; Durante *et al.* 1999). Therefore, unicellular organisms have a variety of sensing mechanisms to adapt cell proliferation rate to variations in nutrient supply (Thevelein & Hohmann, 1995; Teusink *et al.* 1998). One key sensor of this regulation is the glycolytic enzyme pyruvate kinase (E.C. 2.7.1.40). Pyruvate kinase is responsible for the transfer of phosphate from phosphoenolpyruvate to ADP accompanied by formation of ATP and pyruvate. Phosphoenolpyruvate, the substrate of pyruvate kinase is the most energy rich cellular phosphometabolite ( $\Delta G^0 = 58 \text{ kJ}$ ). In comparison,

the  $\Delta G^0$  of ATP is 29 kJ. Therefore, the pyruvate kinase reaction strongly favors the formation of ATP. The Kequilibrium  $\left(\frac{[\text{PEP}] \cdot [\text{ADP}]}{[\text{pyruvate}] \cdot [\text{ATP}]}\right)$  is 1:2000 (pH 8.0, 30°C) (Hess, 1963; Valle *et al.* 1996). Therefore, energy production by pyruvate kinase is more effective than ATP production via mitochondrial respiration. Glycolytic energy production via pyruvate kinase can strongly suppress mitochondrial energy production (Gosalvez *et al.* 1974; Melo *et al.* 1998).

For this reason and since phosphoenolpyruvate and glycolytic phosphometabolites are precursors for synthetic processes, the pyruvate kinase mass action ratio is far removed from its Kequilibrium in all organisms (Hess, 1963; Mazurek *et al.* 1997a, 2001; Zwerschke *et al.* 1999; Bond *et al.* 2000). In unicellular organisms pyruvate kinase is strongly inhibited by ATP and activated by the energy sensor AMP, P<sub>i</sub> and ribose 5-P, the precursors of nucleic

**Abbreviations:** FBP, fructose 1,6-bisphosphate; M2-PK, pyruvate kinase type M2.

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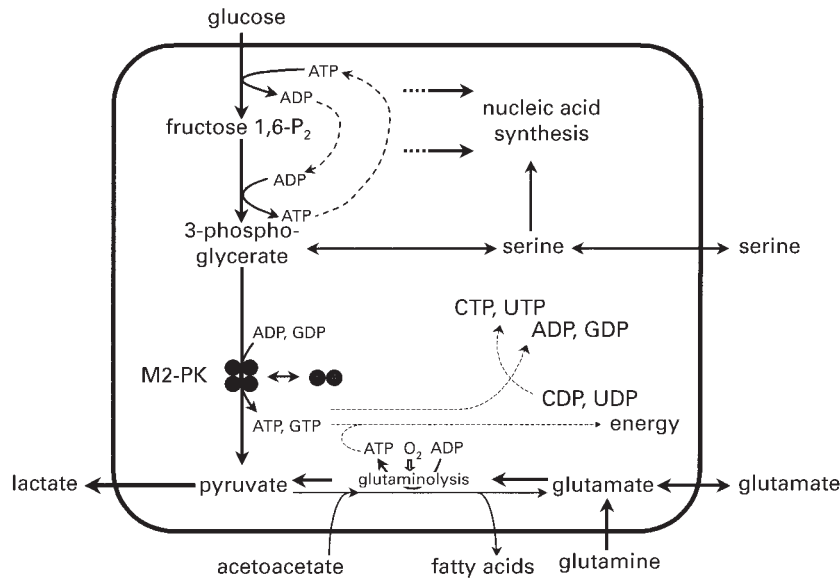


Fig. 1. Regulation of the ATP:ADP and GTP:GDP ratios in tumor cells.

acid synthesis (Fig. 1) (Atkinson & Fall, 1967; Atkinson & Walton, 1967; Maeba & Sanwal, 1968; Tuominen & Bernlohr, 1971; Schramm *et al.* 2000). By this mechanism, pyruvate kinase links energy rich metabolites to the flow of glucose carbons to nucleic acid synthesis, thereby stabilizing the so-called energy charge ( $([ATP]+1/2[ADP]):([AMP]+[ADP]+[ATP])$ ) (Atkinson & Fall, 1967; Atkinson & Walton, 1967). Phosphoenolpyruvate is a precursor of amino acids and complex carbohydrates such as sialic acid (Mazurek *et al.* 1997a). In addition, pyruvate kinase activity is modulated by carbamoyl-P and several different amino acids (Tuominen & Bernlohr, 1971).

The inhibition of pyruvate kinase is essential for synthetic processes and for respiration. Inhibition of pyruvate kinase is highly dangerous when excess carbohydrates (sugars) are available. Glycolysis is organized in such a manner that at first two ATP are consumed for the synthesis of fructose 1,6-bisphosphate before ATP can be regained by the phosphoglycerate kinase (E.C. 2.7.2.3) reaction. Net ATP production occurs in the pyruvate kinase reaction (Thomas & Fell, 1998). In addition, in the glycolytic glyceraldehyde 3-phosphate dehydrogenase reaction NADH is produced. The hydrogen is transferred to pyruvate, the product of the pyruvate kinase reaction and is released as lactate (Eigenbrodt *et al.* 1998a). Thereby, a constant inhibition of pyruvate kinase that favors synthetic processes, can lead to ATP depletion under both conditions: high and low carbohydrate supply (Thevelein & Hohmann, 1995; Mazurek *et al.* 1997b, 1999a; Teusink *et al.* 1998). With an abundance of carbohydrates, ATP is completely consumed for the synthesis of fructose 1,6-bisphosphate. Since unicellular organisms are always in danger of variations in the carbohydrate supply, they have developed a special mechanism to prevent ATP depletion (Thevelein & Hohmann, 1995; Teusink *et al.* 1998). The uptake of carbohydrates from the environment is fueled by phosphoenolpyruvate, the substrate of the pyruvate kinase

reaction. Furthermore, pyruvate kinase is activated by sugar phosphates, especially fructose 1,6-bisphosphate that also regulates the glucose transporter (Thevelein & Hohmann, 1995; Luesink *et al.* 1999). In accordance, in yeast cells, the overexpression of pyruvate kinase leads to an inhibition of cell proliferation by depletion of glycolytic phosphometabolites. The overexpression of the protein kinase MCKinase that phosphorylates and inactivates pyruvate kinase restores cell proliferation. Besides the inhibition of cell proliferation, the overexpression of pyruvate kinase also blocks the formation of spores (Brazill *et al.* 1997). Spores allow the survival of unicellular organisms under nutrient limitation and unfavourable external conditions for a longer time. Sporulation is linked to another phosphometabolite, 3-phosphoglycerate, which is accumulated in spores in a great amount (Nelson & Kornberg, 1970a, b; Rhaese *et al.* 1976). In contrast to ATP, fructose 1,6-bisphosphate and phosphoenolpyruvate, 3-phosphoglycerate is highly stable but a low energy compound of the glycolytic sequence. However, under the catalysis of phosphoglyceromutase (E.C. 2.7.5.3) and enolase (E.C. 4.2.1.11), 3-phosphoglycerate is converted to energy rich phosphoenolpyruvate, the substrate of the ATP producing pyruvate kinase. During resporulation the first event is the activation of these three enzymes and the upregulation of the flow of 3-phosphoglycerate to pyruvate to restore NTP pools (Nelson & Kornberg, 1970a, b). By linking energy metabolism with synthetic (anabolic) processes, pyruvate kinase dependent on nutrient supply, regulates cell proliferation, sporulation and cell death.

In recent years it has been shown that the same central role of pyruvate kinase in concert with other glycolytic enzymes is found in multicellular organisms, where nutrient supply is more or less constant. Studies with mammals have shown that also in these species, pyruvate kinase is involved in fundamental processes such as cell proliferation, differentiation, tumor formation and apoptosis (Mazurek *et al.* 1997a, 1999a).

## The role of pyruvate kinase isoenzymes in multicellular organisms

In multicellular organisms such as mammals only tumor cells and spermatides have comparably high levels of glycolytic phosphometabolites to those found in active proliferating unicellular organisms (Boscá & Corredor, 1984; Eigenbrodt *et al.* 1985). In differentiated tissues phosphometabolite levels are 100-fold lower than in proliferating cells. One exception is glycerate 2,3-bisphosphate which is low in tumor cells and high in differentiated tissues, especially erythrocytes. In normal proliferating cells glycolytic phosphometabolites rise 10-fold in the G1-phase, but never reach levels as high as in tumor cells (Chesney *et al.* 1999; Aulwurm & Brand, 2000; Perez *et al.* 2000). The isoenzyme of pyruvate kinase that allows the upregulation of phosphometabolite pools in multicellular organisms is pyruvate kinase type M2 (M2-PK). M2-PK is an ancient variant of the pyruvate kinase enzyme found in unicellular organisms such as yeast and *E. coli* (Eigenbrodt *et al.* 1985, 1992; Valle *et al.* 1996; Bond *et al.* 2000; Schramm *et al.* 2000). Since M2-PK is found in tissues with a high capacity for nucleic acid synthesis, we have termed those tissues *nucleogenic tissues* to differentiate them from *gluconeogenic tissues* with a high rate of gluconeogenesis and from *lipogenic tissues* with a high rate of lipogenesis (Mazurek *et al.* 1997a).

In differentiated tissues pyruvate kinase isoenzymes other than M2-PK are found: M1-PK is characteristic for tissues with high rates of energy consumption and low phosphometabolite pools such as muscle and brain. Pyruvate kinase type L is found in gluconeogenic tissues such as liver and kidney. In erythrocytes pyruvate kinase type R is expressed. The different pyruvate kinase isoenzymes react to individual allosteric regulators (Eigenbrodt *et al.* 1994). They are phosphorylated by different specific protein kinases and are associated with different other enzymes as well as proteins with non-glycolytic functions such as actin, tubulin or band 3 protein (Eigenbrodt *et al.* 1994). The regulation of pyruvate kinase activities by association with other enzymes and proteins seems to be a very ancient phenomenon, since in bacteria the *E-ras* protein and nucleotide diphosphate kinase selectively bind to pyruvate kinase thereby modulating the ATP:ADP and GTP:GDP ratios (Chopade *et al.* 1997). Associations of different glycolytic enzymes with other proteins have also been found in mammalian cells. To demonstrate the association between glycolytic enzymes and other proteins different methods are used, e.g. coimmunoprecipitation and the two-hybrid technique (Zwerschke *et al.* 1999). We have developed a method to determine the association of several glycolytic enzymes and could show that not only the glycolytic enzymes but also RNA, oncoproteins and components of the protein kinase cascade can be associated in a so-called *glycolytic enzyme complex* (Mazurek *et al.* 1996, 1998, 1999b, 2001). Demonstration of the glycolytic enzyme complex is based on the isoelectric points of the proteins. Proteins associated within the glycolytic enzyme complex focus at a common isoelectric point that is different from the isoelectric point of the purified individual proteins. Migrations of proteins inside and

outside the glycolytic enzyme complex are reflected by shifts in their isoelectric points. The glycolytic enzyme complex has mainly been found perinuclear but some of the enzymes are also bound to the cytoskeleton, mitochondria or in the nucleus (Mazurek *et al.* 1997a, b; Bereiter-Hahn *et al.* 1998; Engel *et al.* 1998; Popanda *et al.* 1998; Nguyen *et al.* 2000). The association of enzymes and other compounds within the glycolytic enzyme complex leads to a compartmentalization of metabolic processes. The enzyme composition of the glycolytic enzyme complex controls phosphometabolite pools and the interaction between glycolysis, glutaminolysis and serinolysis (Mazurek *et al.* 2001).

M2-PK occurs in a highly active tetrameric form with a high affinity to its substrate phosphoenolpyruvate and in a less active dimeric form with a low PEP affinity. Only the tetrameric form of M2-PK is associated within the glycolytic enzyme complex (Mazurek *et al.* 1997b; Zwerschke *et al.* 1999).

During tumorigenesis the tissue specific isoenzyme generally disappears and M2-PK is expressed. In tumor cells generally the dimeric form of M2-PK is dominant and is released into the blood (Oremek *et al.* 1999). Therefore, we have termed the dimeric form of M2-PK as *tumor M2-PK* (Eigenbrodt *et al.* 1992). The dissociation of the tetrameric form to the dimeric form is induced by oncoproteins. The first oncoprotein that was found to interact with M2-PK was the activated pp60<sup>v-src</sup> kinase. The pp60<sup>v-src</sup> kinase phosphorylates M2-PK on a tyrosine residue (Presek *et al.* 1988; Eigenbrodt, *et al.* 1998b). Another oncoprotein that leads to a dimerization of M2-PK is the HPV-16 E7 oncoprotein that directly binds to M2-PK. The interaction of the oncoproteins with M2-PK always leads to an accumulation of all phosphometabolites above pyruvate kinase which are then channeled to synthetic processes, e.g. nucleic acid synthesis.

Besides the expansion of phosphometabolite pools, the dimerization of M2-PK has another important consequence that concerns energy metabolism. As in unicellular organisms, in mammals both glucose limitation and oversupply of glucose, lead to imbalances in the ATP:ADP ratio, to an inhibition of pyruvate kinase and can cause cell death (Live & Kaminskas, 1975; Mazurek *et al.* 1997a, b, 1999a; Wang & Iynedjian, 1997). Therefore, tumors with a high glycolytic flux rate are sensitive to glucose limitations (Mazurek *et al.* 1997b; Shim *et al.* 1998). Presumably, to prevent cell death by glucose limitation tumor cells have reduced mitochondrial pyruvate consumption and have activated a pathway that produces pyruvate and energy from the amino acid glutamine. This pathway has been termed as *glutaminolysis* in analogy to glycolysis, see Fig. 1 (McKeehan, 1982). In contrast to glycolysis, glutaminolysis is dependent upon oxygen supply (Eigenbrodt *et al.* 1998a). Both pathways are active in proliferating and lactating mouse mammary gland where the synthetic products of glycolysis such as fructose 1,6-bisphosphate and nucleotides are released into the milk. During mouse mammary gland apoptosis both pathways are simultaneously inactivated which leads to a drastic drop in the nucleotide triphosphate pools (Mazurek *et al.* 1999a).

Investigations on human tumors xenografted in nude rats have revealed that besides glutamine the amino acid serine is used in great amounts as a substrate for pyruvate production. Serine is converted to 3-phosphoglycerate and further converted to pyruvate and lactate with the formation of ATP. This pathway has been termed *serinolysis* in analogy to glycolysis and glutaminolysis (Eigenbrodt *et al.* 1998a; Mazurek *et al.* 2001). In addition, serine can expand pyruvate pools when the flow through the glyceraldehyde 3-phosphate dehydrogenase (E.C. 1.2.1.12) reaction is limited. In solid tumors the flow of serine to lactate is highest when the glucose carbons are predominantly used for nucleic acid synthesis. Investigations of transformed NIH 3T3 cells revealed that the organization of the glycolytic enzyme complex plays an important regulatory role in this process. Only the tetrameric form of M2-PK is associated within the glycolytic enzyme complex. The dimeric form is located outside the complex. Transformation of a low glycolytic NIH 3T3 cell line which is characterized by a large amount of the dimeric form of M2-PK, high phosphometabolite levels and a high proliferation rate is only possible when phosphoglyceromutase reintegrates into the glycolytic enzyme complex (Mazurek *et al.* 2001). Thereby, the flow of serine to 3-phosphoglycerate, pyruvate and lactate increases. The glycolytic enzyme complex associated form of phosphoglyceromutase is the histidine phosphorylated form that is independent of glycerate 2,3-bisphosphate. The phosphorylation and activation of phosphoglyceromutase is presumably caused by a direct transfer of phosphate from the histidine of the enzyme nucleotide diphosphate kinase to the histidine of phosphoglyceromutase. This might be an explanation of why nucleotide diphosphate kinase is constantly altered during tumorigenesis (Mazurek *et al.* 1998).

### Fatty acids and glutamate, two alternatives for hydrogen release

Due to an upregulation of glycolytic phosphometabolites, tumor cells usually contain no active glycerol 3-phosphate shuttle or only very low activities of glycerol 3-phosphate dehydrogenase (E.C. 1.1.1.8). Therefore, in tumor cells the hydrogen produced in the glycolytic glyceraldehyde 3-phosphate dehydrogenase reaction is transferred to pyruvate and released as lactate. In addition, tumor cells use two further pathways to export the hydrogen (Mazurek *et al.* 1997b).

Investigations with tumor cells and solid tumors have shown that tumor cells have the enzymatic and metabolic capacity for *de novo* synthesis of all fatty acids, whereby saturated and monounsaturated fatty acids are predominantly formed and released (Fig. 2) (Punnonen *et al.* 1989; Pizer *et al.* 1998). This explains why in contrast to normal tissues the membrane of tumor cells predominantly contains saturated and monounsaturated fatty acids (Punnonen *et al.* 1989; Lee *et al.* 1995). For *de novo* synthesis of fatty acids, acetyl CoA and NADPH are necessary. NADPH is produced in large amounts by the NADP<sup>+</sup> dependent malate decarboxylase (E.C. 1.1.1.40) and the NADP<sup>+</sup> dependent isocitrate dehydrogenase (E.C. 1.1.1.42). Both are strongly over-expressed in human tumors (Mazurek *et al.* 2000). The oxidative pentose phosphate cycle, the alternative NADPH producing pathway, is blocked by the high fructose 1,6-bisphosphate levels found in tumor cells. Therefore, in tumor cells the mass of ribose 5-phosphate is synthesized by the transketolase-transaldolase reactions (Boros *et al.* 1997; Lee *et al.* 1998).

In solid tumors, acetyl CoA derives from ketone bodies (acetoacetate and  $\beta$ -hydroxybutyrate) and glucose carbons. A good correlation was found between glucose consumption and

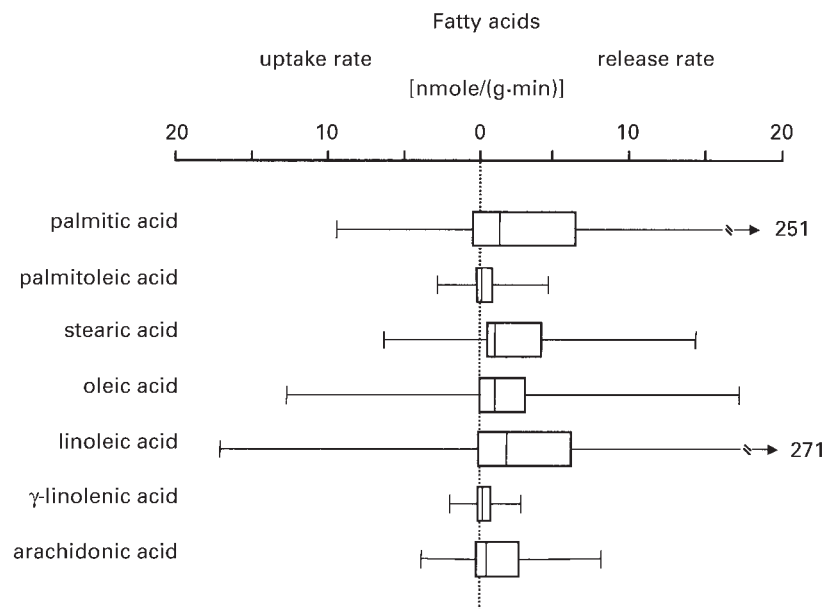
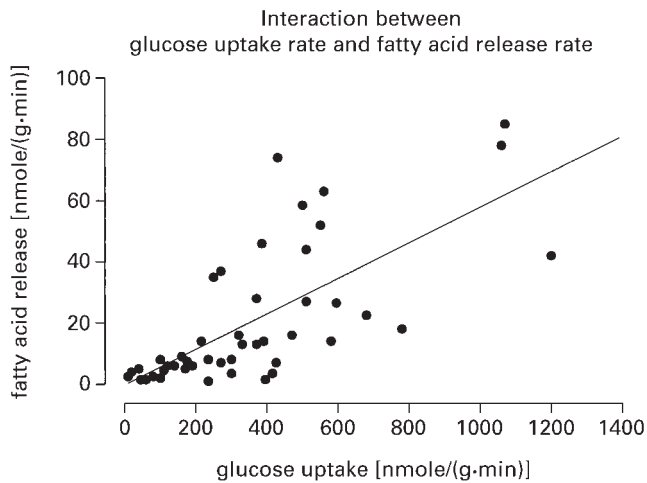
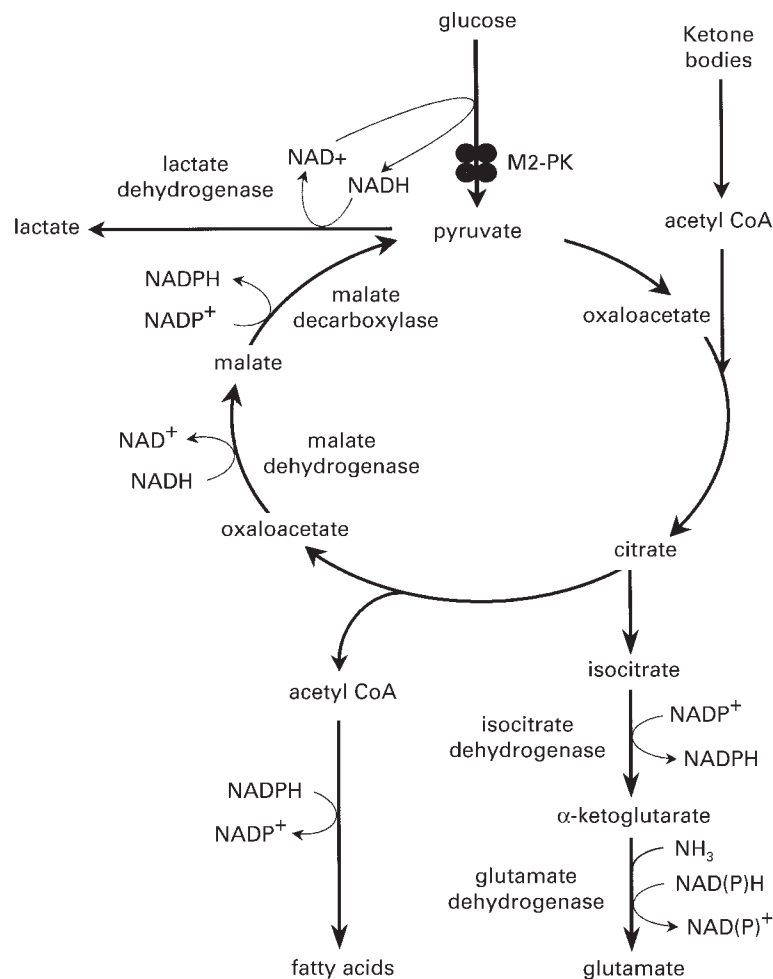


Fig. 2. Fatty acid conversion rate in xenotransplanted human mammary carcinomas,  $n = 66$ .



**Fig. 3.** Interaction between glucose uptake rate and fatty acid release rate in xenotransplanted human mammary carcinoma. Slope=58, intercept= - 0.25 nmole/(g·min),  $r = 0.706$ .

lactate production by tumors and between glucose consumption and fatty acid release rate (Fig. 3) (Kallinowski *et al.* 1987, 1988; Vaupel *et al.* 1987; Eigenbrodt *et al.* 1998a). In the mitochondria oxaloacetate and acetyl CoA condense to form citrate that is transported into the cytosol. In the cytosol citrate can be converted to glutamate and is released as glutamate or glutamine (Fig. 4). A greater part is converted to acetyl CoA and oxaloacetate by ATP citrate lyase. Oxaloacetate is further converted to malate and pyruvate by malate dehydrogenase and the  $\text{NADP}^+$  dependent malate decarboxylase. Acetyl CoA and the  $\text{NADPH}$  produced in the malate decarboxylase reaction are channeled to fatty acid synthesis (Fig. 4). The fatty acids produced can be used for phospholipid synthesis or can be released from the cell. Glutamate, alanine, glycine and fatty acid released from tumors are able to suppress immune cell functions (Eck *et al.* 1989; Grimm *et al.* 1994, 1995; Jiang *et al.* 1998; Wheeler *et al.* 1999). Part of the acetate for the synthesis of glutamate derives from ketone bodies. Glutamate can be converted to glucose in liver and kidney. This may explain why tumor patients become cachectic without developing



**Fig. 4.** Hydrogen release in tumor cells. In tumor cells one important way to release the hydrogen produced in the glycolytic glyceraldehyde 3-phosphate dehydrogenase reaction is the transfer to pyruvate and the release as lactate. Alternatively, the hydrogen can also be exported as glutamate or as fatty acids.

hypoglycaemia or ketosis. Saturated and monounsaturated fatty acids inhibit 6-phosphofructo 1-kinase and M2-PK (Marchut *et al.* 1986). Therefore, in the future it will be interesting to learn whether those fatty acids can inhibit tumor growth by interfering with glycolysis.

Tumor cells and immune cells are characterized by similar metabolic features such as high glycolytic and glutaminolytic flux rates which allow them to invade areas with low levels of oxygen and glucose. It will therefore be very important to develop new immunotherapeutical approaches to learn more about the metabolic interference of both cell types (Sebolt & Weber 1984; Brand *et al.* 1987; Newsholme & Calder, 1997; Kew *et al.* 1999).

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