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Hexokinase Binding to Mitochondria: A Basis for Proliferative Energy Metabolism

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Current thought is that proliferating cells undergo a shift from oxidative to glycolytic metabolism, where the energy requirements of the rapidly dividing cell are provided by ATP from glycolysis. Drawing on the hexokinase–mitochondrial acceptor theory of insulin action, this article presents evidence suggesting that the increased binding of hexokinase to porin on mitochondria of cancer cells not only accelerates glycolysis by providing hexokinase with better access to ATP, but also stimulates the TCA cycle by providing the mitochondrion with ADP that acts as an acceptor for phosphoryl groups. Furthermore, this acceleration of the TCA cycle stimulates protein synthesis via two mechanisms: first, by increasing ATP production, and second, by provision of certain amino acids required for protein synthesis, since the amino acids glutamate, alanine, and aspartate are either reduction products or partially oxidized products of the intermediates of glycolysis and the TCA cycle. The utilization of oxygen in the course of the TCA cycle turnover is relatively diminished even though TCA cycle intermediates are being consumed. With partial oxidation of TCA cycle intermediates into amino acids, there is necessarily a reduction in formation of CO_2 from pyruvate, seen as a relative diminution in utilization of oxygen in relation to carbon utilization. This has been assumed to be an inhibition of oxygen uptake and therefore a diminution of TCA cycle activity. Therefore a switch from oxidative metabolism to glycolytic metabolism has been assumed (the Crabtree effect). By stimulating both ATP production and protein synthesis for the rapidly dividing cell, the binding of hexokinase to mitochondrial porin lies at the core of proliferative energy metabolism. This article further reviews literature on the binding of the isozymes of hexokinase to porin, and on the evolution of insulin, proposing that intracellular insulin-like proteins directly bind hexokinase to mitochondrial porin.

KEY WORDS: Cancer; proliferation; Crabtree effect; insulin action; compartmentation; aerobic glycolysis; hexokinase; mitochondria; porin; protein synthesis; TCA cycle.

The increased binding of the type II isoenzyme of hexokinase (HKII) to porins on mitochondria of proliferating cells has been a subject of great scientific interest and endeavor over the last decade. Interesting discoveries have been made in isolating, sequencing, characterizing, and investigating the genetics of both HKII and the mitochondrial porin molecules. However, the role of the increased HK–mitochondrial binding in proliferative metabolism has not been clearly outlined.

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The hexokinase–mitochondrial acceptor theory of insulin action, proposed in the 1960s,[1–3] is a model which unifies all of the diverse anabolic functions of insulin, and can throw light upon the metabolic consequences of HKII binding to mitochondria in proliferating cells.[4] According to this model, insulin fulfills its roles by directly binding HK to mitochondrial porin, and stimulating mitochondrial respiration. The enhancement of the rate of the TCA cycle by insulin has been found to accelerate protein synthesis.[5]

The needs for energy production and protein synthesis are clearly fundamental in a rapidly proliferating cell. Current thought is that the energy requirements

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of the proliferating cell are provided by ATP from glycolysis, following a shift from oxidative to glycolytic metabolism in proliferating cells. Evidence is presented in this article suggesting that despite the markedly increased rate of glycolysis, the TCA cycle is still being stimulated, in fact by the very binding of the HK to the mitochondria. However, the increased binding coincident with the reductive anabolic metabolism of proliferation results in only a trivial acceleration of the net cellular oxygen consumption. In light of the mechanism of compartmentation proposed for mitochondria stimulated by insulin,[6] it is suggested that the mitochondria whose TCA cycle is stimulated by increased HK–porin binding in proliferating cells provide ATP specifically to anabolic pathways whose enzymes are directly adjacent to these mitochondria.

According to the HK–mitochondrial acceptor theory, the insulin molecule enters all insulin-sensitive cells, i.e., muscle and adipose tissue, and causes hexokinase, the first enzyme of the glycolytic pathway, to bind to the mitochondrial membrane, in proximity to the porins. Porins, also known as voltage-dependent anion channel isoforms (VDACs), are protein channels through which ATP generated within mitochondria moves out to enter the cytosol.[7] This positioning of HK by insulin provides HK with better access to mitochondrial ATP, so that glucose can be phosphorylated more expeditiously. In the meantime, ADP formed by hexokinase outside the mitochondrion goes back into the mitochondrion, where it stimulates ATP production by acting as an acceptor for phosphoryl groups. By providing an "acceptor effect," insulin accelerates mitochondrial respiration (Figs. 1a, 1b).

There are many lines of evidence in support of the HK–mitochondrial acceptor theory of insulin action. That insulin is internalized and acts through intracellular sites to stimulate protein synthesis has been established by Miller and Sykes.[8] The localization of HK on the mitochondrial membrane and adjacent to porin molecules has been established by various studies in rat liver, brain, and kidney mitochondria.[9,10] The effect of insulin on the binding of HK to the mitochondrial membrane is supported by several lines of evidence, including the finding of an inverse relationship between the effectiveness of insulin action on various tissues and the percentage of HK bound to the mitochondria in these tissues,[11,12] the release of HK from mitochondria of lactating mammary glands upon their exposure to antibodies against insulin[13]; the increased binding of HK to the mitochondria in fat pads incubated with insulin[14]; and the inability of

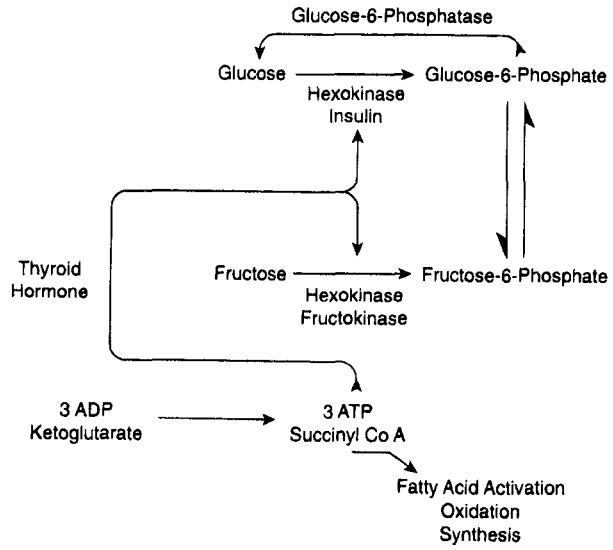

Fig. 1a. Redrawn from the original diagram describing the HK–mitochondrial acceptor theory of insulin action. The site of insulin action is hypothesized to be hexokinase, an enzyme which accelerates the Krebs cycle by providing acceptors for high-energy phosphate produced in this cycle. Taken from Ref. 1.

insulin to stimulate protein synthesis in diaphragm muscle whose HK pathway is interrupted.[15] In a number of tissues, it has been documented that ATP generated in the mitochondrion by oxidative phosphorylation, activated by the return of ADP into the mitochondrion, is more accessible to the mitochon-

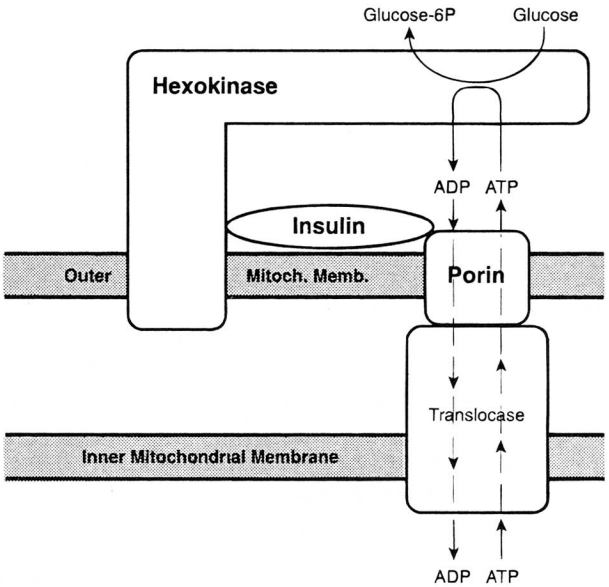

Fig. 1b. The proposed role of insulin, or an insulin-like protein, in attaching hexokinase to porin on the outer mitochondrial membrane.

drion–bound HK than are extracellular sources of ATP.[16] In addition, the critical role of mitochondrial ATP synthesis in insulin action is documented by the loss of insulin effect on muscle or liver glycogen synthesis under anaerobic conditions,[5] the loss of insulin-stimulated transport in a muscle cell line following treatment of the muscle cells with dinitrophenol,[17] and the loss of insulin's enhancing effect on the transport of amino acids in anaerobiosis.[18]

It has been proposed that a major biological mechanism fueling neoplastic growth is the establishment of a vicious cycle where oncoproteins cause an increase in cellular protein synthesis, hence causing translation of more oncoproteins.[19] As reviewed by Rosenwald,[19] when resting cells are stimulated by mitogens, a net increase in protein synthesis is noted prior to entrance into the S phase of the cell cycle.[20,21] In *ras* transformed fibroblasts, a fourfold increase in the rate of protein synthesis has been described.[22] In one study with polyoma virus-transformed rat embryo fibroblasts, the total protein synthesis and accumulation was noted to be higher in the transformed cells than in normal parental cells, while the rate of the degradation of proteins remains almost unchanged.[19]

In elegant studies using differently ^{14}C labeled glucose in mitogen-stimulated thymocytes, Greiner *et al.* have noted interesting findings on the fate of the carbons of glucose during the transition from rest to proliferation.[23] They have established that $^{14}CO_2$ production from 1-^{14}C glucose increases by approximately 2 times during proliferation, whereas $^{14}CO_2$ production from 3,4-^{14}C glucose remains constant. However, $^{14}CO_2$ production from 6-^{14}C glucose decreases by more than 50%. What could explain the differential fates of the carbons of glucose molecule during the transition from rest to proliferation?

It has been well documented that the binding of hexokinase to mitochondria by insulin causes a stimulation of protein synthesis.[5] In studies using ^{14}C labeled pyruvate, it has been shown that insulin preferentially stimulates carbons 2 and 3 of pyruvate to be incorporated into protein.[24] The carboxyl group of pyruvate, which is lost before the acetyl group enters the Krebs cycle, is unaffected by insulin. These findings have confirmed the notion that insulin stimulates conversion to protein mainly of the carbons that pass through the mitochondrial Krebs cycle.

Many of the findings on the fate of the carbons of glucose during transition to proliferation effectively duplicate the effects of insulin on the fate of the carbons of glucose. The fact that $^{14}CO_2$ production from 3,4-^{14}C glucose remains constant in the transition from rest to proliferation, is parallel to the effect of insulin on the carbons of pyruvate. Carbons 3 and 4 of glucose correspond to carbon 1 of pyruvate. It was noted that insulin does not stimulate incorporation of the first carbon of pyruvate into protein since this carbon is removed by decarboxylation prior to the molecule's entry into the Krebs cycle. Furthermore, carbons 1 and 6 of glucose correspond to carbon 3 of pyruvate. Studies by Mohan and Bessman[24] have shown that carbon 3 of pyruvate is preferentially stimulated by insulin to be incorporated into protein. The decrease in CO_2 production from carbon 6 of glucose in proliferation would be explained if the TCA cycle is stimulated and more of the 6 carbons are diverted into protein, so less would be left to be oxidized into CO_2. One may then question how is it that in the mitogen-stimulated cells carbons 1 and 6 of glucose have different fates as a result of transition to proliferation, if both of these correspond to carbon 3 of pyruvate? In other words, why would the ^{14}C-CO_2 production from 6-^{14}C decrease by half, but from 1-^{14}C double? This can be clearly understood by noting that the first carbon of glucose can be decarboxylated to produce CO_2 by the pentose phosphate shunt that would indeed be required to provide ribose for the increased nucleic acid requirements.

Hence, the HK binding to mitochondria in proliferating cells stimulates protein synthesis by two mechanisms. First, by stimulating ATP production from both glycolysis and TCA cycle. Second, by bringing the protein synthesis machinery a faster provision of amino acids obtained from pyruvate and the intermediates of the TCA cycle. Of note, alanine, aspartate, and glutamate are either reduction or partially oxidized transaminated products of the intermediates of the TCA cycle and glycolysis, pyruvate, oxaloacetate, and α-ketoglutarate, respectively.

The role of the high rate of aerobic glycolysis in the energy metabolism of proliferating and tumor cells has been a mystery. When the high rate of lactate formation from glucose in the presence of oxygen was noted in tumor cells by Warburg some 60 years ago, Warburg postulated that there must be a respiratory defect in these cells, whereby glucose cannot be fully oxidized to CO_2.[25] Crabtree later provided evidence suggesting that the addition of glucose to tumor cells produced an approximate 10% diminution in oxygen consumption.[26] Over the years, various factors, including an alteration in the control of glycolysis via induction of various glycolytic enzymes, alterations in

the activity of ATPase, and failures of NADH transport into mitochondria (reviewed in Ref. 23) have been considered as potential contributors to aerobic glycolysis in proliferating cells. Most recently, studies in mitogen-stimulated rat thymocytes have suggested that it is not limitation of TCA cycle, nor decreased ATP supply, that causes decreased glucose oxidation in these cells. It is suggested that glucose induction of glycolytic enzymes causes an increased regeneration of NAD^+ in the cytoplasm during proliferation, and that this competes with NADH transport into mitochondria, and subsequently with its oxidation.[23]

In their experiments studying the interaction between glycolytic and oxidative metabolism in cells undergoing transition from rest to proliferation using mitogen-activated thymocytes, Greiner *et al.*[23] have noted that glucose utilization and lactate formation increase 18- and 38-fold, respectively, with proliferation. However, the absolute amount of $^{14}CO_2$ production by pyruvate dehydrogenase remains constant, while $^{14}CO_2$ production by the TCA cycle is decreased during transition from rest to proliferation. These findings would suggest a shift from oxidative to glycolytic energy production. In glycolysis only 2 molecules of ATP are produced from each molecule of glucose, as compared to 36 molecules of ATP when the glucose molecule is completely oxidized to CO_2. This shift would be an inefficient mechanism of providing energy to a rapidly dividing cell.

HK is the first enzyme of glycolysis and catalyzes one of the three steps in the glycolytic pathway which involve large negative free energy changes, and are therefore effectively irreversible. When HK is bound to the porin on a mitochondrion, it is provided with preferential access to ATP produced within the mitochondrion,[27] and is thus stimulated. This can make a major contribution to the increased rate of glycolysis noted under aerobic conditions in proliferating cells. A relative decrease in CO_2 production in face of increased glucose utilization and lactate formation noted during transition from rest to proliferation should not, however, mean a diminution in the TCA cycle activity. In fact, a stimulated TCA cycle is evidenced by the differential fates of the carbons of glucose from rest to proliferation. Much of the problem surrounding the interpretation of the Crabtree effect is based on the fact that the turnover of the TCA cycle has been assumed to be revealed by oxygen consumption, and thus it has been assumed that the TCA cycle is slowed down with proliferation, when in reality the anabolic role of the TCA cycle in providing amino acids for protein synthesis is reflected as a diminution in oxygen uptake. With partial oxidation of TCA cycle intermediates into amino acids, there is necessarily a reduction in formation of CO_2 from pyruvate, seen as a relative diminution in utilization of oxygen in relation to carbon utilization. Hence a shift from oxidative to glycolytic metabolism has been assumed (the Crabtree effect). In addition, the net overall metabolism during proliferation must be anabolic and hence reductive in nature. Large numbers of carbons are bound together to form long-chain fatty acids to be used in lipoproteins of cell membranes, for example, instead of being oxidized to CO_2, hence further decreased oxygen utilization and CO_2 production with proliferation. Indeed, observing the dramatic increase in lactate production and aerobic glycolysis in proliferating cells, one needs to ask: *Where does the lactate go?* Does it all leave the proliferating cell and enter circulation? The lactate can in fact be made back into pyruvate, which can either enter the TCA cycle and contribute carbons to increased protein synthesis, or, it can be made into Acetyl-coA, and fall into the pathway of lipid synthesis.

It has been noted that the respiration and the oxidative phosphorylation as well as the energy charge and the NADH/NAD ratios in tumor cells are not significantly different from normal cells (reviewed in Ref. 28). If insulin does in fact carry out its function also by binding HK to mitochondria, one may understand the above by drawing a parallel to the case of cellular response to insulin. Considering that insulin causes a 20–30% acceleration of most anabolic reactions, for a long time it was not understood why insulin has only a trivial effect on the total oxygen consumption of tissue. This was explained by Bessman *et al.* as follows.[5] Most of the ATP produced in the cell is consumed in maintenance reactions like the Na^+,K^+-ATPase activity which are not substantially influenced by insulin. The anabolic activities of the cell such as protein and glycogen synthesis consume less than 10% of the total ATP produced by the resting cell.[29] It has been previously suggested that insulin acts on a small fraction of the mitochondrial Krebs cycle that is directly coupled to providing ATP for synthetic reactions and substrate transport.[5] In the proliferating transformed cell, one may presume the increased binding of HKII to the porin occurs specifically on mitochondria adjacent to the apparatus for protein synthesis required for cell division.

There is evidence in favor of the stimulation of the mitochondrial Krebs cycle in proliferating cells. It has been noted that the addition of glucose to respiring

hepatoma mitochondria results in stimulation of respiration, but has no effect on the respiration of mitochondria from control and regenerating liver cells.[30] There is also a number of adenocarcinoma cells noted to be characterized by respiratory activity higher than that of normal epithelial cells.[31] Furthermore, in studies using rhodamine 123, a lipophilic cationic compound that is taken up by mitochondria with high membrane potential, the effects of PDGF on mitochondrial bioenergetics of BALB-c3T3 cells has been investigated. These studies have shown that PDGF has a stimulatory effect on the bioenergetics of resting cells.[31] Interestingly, it has also been noted that within 3 hours of PDGF binding to its receptor on the cell membrane of these cells the mitochondria become localized more to the perinuclear area,[32] where it has been suggested the ATP generated within the mitochondria is more accessible to the enzymes of DNA synthesis within the nucleus.

If the binding of HK to mitochondria does in fact play a critical role in providing the transformed cell with both its protein and energy requirements, then a good understanding of the HK molecule structure, function, and regulation of expression, in addition to an understanding of the biochemistry of the porin molecule, would be indispensable to finding a mechanism to specifically stop this binding in transformed cells. Hexokinases are present in all cells and are clearly fundamental to energy metabolism. In mammalian tissues, HK is present in four different isoenzyme forms which have different kinetic properties and are designated as types I, II, III, and IV (glucokinase), based on their electrophoretic mobility. The ratio of the four isoenzymes in normal tissues appears to be correlated with their rate of glucose consumption.[33] HKI is the predominant type in the brain and in erythrocytes, both of which depend strictly on circulating glucose. Type II is the predominant type in insulin-sensitive tissues, such as skeletal muscle, heart, diaphragm, and adipose tissue. This type is also the predominant isoenzyme in various tumor cells and is the focus of the discussion in this article. Type III hexokinase is found in small amounts in some adult tissues, where its expression is also constitutive like Type I,[34] and Type IV hexokinase, or glucokinase, is by far the most abundant isozyme in normal liver cells.

The types I, II, III and the tumor hexokinases all consist of a single polypeptide chain of approximately 100 kDa, whereas the Type IV (glucokinase) has a molecular weight of about 50 kDa. Cloning and sequencing studies of hexokinase from a variety of tissues have led to the notion that the 100-kDa isozymes have evolved from the gene duplication, gene fusion of an ancestral 50-kDa gene. Hexokinase is a regulatory enzyme which is allosterically inhibited by its products, glucose-6-phosphate and ADP. Hexokinase can phosphorylate various hexoses, and its K_m lies at a glucose concentration of 0.1 mM.

Understanding the differences in the binding of the different isoenzymes of hexokinase to mitochondria may help to throw light upon the exact nature of the binding of HKII to the porins in transformed cells. Among the four isoenzymes of HK, HK I and II are capable of reversibly binding to mitochondria and type IV is largely in the soluble form.[35] Type III HK has been found localized to the nuclear membrane in several tissues.[36] Type II HK has been shown to increase its contact with mitochondria in skeletal muscle and heart upon stimulation with insulin.[37,38] However, in the brain where the predominant HK is Type I, there is no such response to insulin as nearly all of the HK is already bound to the mitochondria.[39] The exact nature of the binding is not clear. Studies of the structure and function of HK I–III have shown that the C-terminal half of the enzyme has both catalytic and regulatory roles, while the amino terminal may be involved in membrane binding.[40] In contrast to the glucokinase in the liver, glucokinase in beta cells is bound to mitochondria. This tissue specific difference has been suggested to be the result of differential splicing of mRNA coding for the amino terminus.[41] The amino terminal 15 amino acid sequence of HK I is predicted to be an α-helical structure. This domain is longer than the amino terminal domain of yeast and has been shown to be necessary and sufficient for HKI binding to porin.

In Morris hepatoma 3924A, HKII is the predominant HK isoenzyme. Two fractions of HK, i.e., IIa and IIb, were isolated from both the soluble and mitochondria-enriched fractions of these cells using chromatographic studies.[33] The Type IIb was the predominant type in both fractions, but the IIb/IIa ratio was higher in the particulate fraction. It was suggested that the greater hydrophobicity and lower negative charge of the type IIb in comparison with IIa may explain the increased binding of this to the mitochondria.[33] Whereas the N-terminal region of HK has been shown to include the only hydrophobic peptide sequence in the brain HK, it has been suggested that maybe the difference in the hydrophobicity indicates differences in the N terminal region. Whereas two distinct mRNA transcripts for HKII were found in these cells, it was

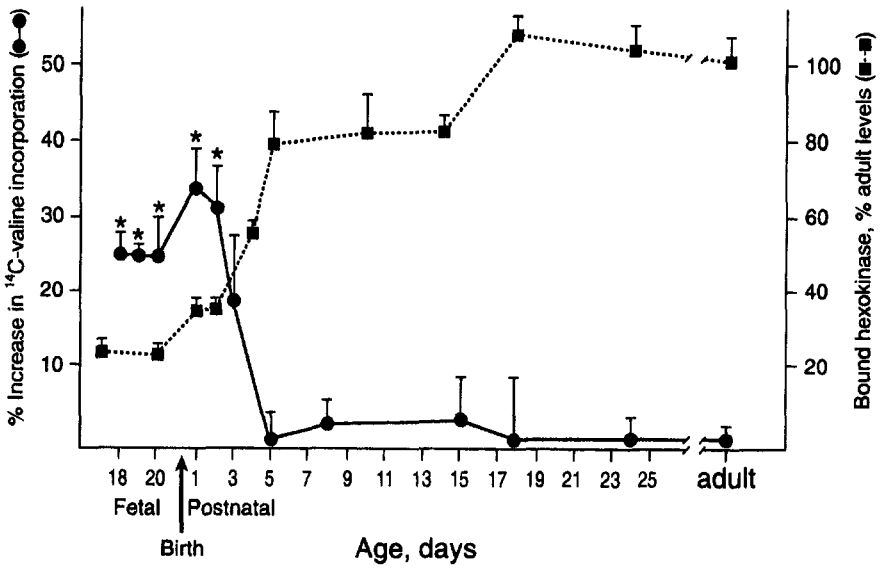

Fig. 2. Effect of insulin on brain protein synthesis related to bound hexokinase activity in the developing rat brain cortex. (•) Percent increase in ^{14}C-valine incorporation into rat brain with insulin (10 mU/ml); (■) total mitochondria-bound hexokinase, expressed as percent of adult levels. Taken from Ref. 47.

suggested that perhaps the microheterogeneity is due to differential RNA transcription or processing. HKII microheterogeneity has been found in normal and tumor cells.[33] A detailed analysis of the HK gene in rat adipose tissue showed that two transcripts were synthesized from one transcription initiation site and two different termination sites.[34] The significance of the presence of the two subtypes is not yet clear.

The insulin molecule is an ancient protein, a member of a superfamily of growth factors and hormones which includes insulin-like growth factors, relaxin, and the silkworm prothoracicotrophic hormone.[42] Although the insulin molecule itself has been highly conserved throughout the vertebrates from the hagfish to the birds and mammals,[43] insulin-like molecules have been found in multicellular nonvertebrates, including insects, molluscs, echinoderms, flowering plants, and even unicellular organisms.[44] Considering the extensive distribution of insulin-like peptides, it has been suggested that insulin-like proteins might have evolved from a serine family of proteases,[45] and that the insulin molecule had its more recent origin as a neuropeptide.[43]

There is a large body of evidence for the synthesis of insulin-like proteins in the CNS.[46] However, the function of this intracellular insulin-like protein has been a mystery. It is known that insulin has no effect on adult brain tissue. However, one of the first nerve growth factors discovered by Levi-Montalcini was an insulin-like protein which stimulated growth of fetal tissue nerve cells in culture, and elicited in these cells the metabolic effects seen after insulin treatment. In studies of rat brain, the effect of insulin in stimulating protein synthesis is observed up to the 4th day of postnatal life. Thereafter the brain tissue becomes completely insensitive to insulin.[47] The stimulatory effect of insulin on ^{14}C-valine incorporation into brain protein is inversely proportional to the binding of hexokinase to mitochondria in these cells (Fig. 2). It must be that there is a surge in the expression of an intracellular insulin-like protein starting just about the time of birth, and maximizing by the 5th day of postnatal life in the rat brain. This fact that the adult brain is not sensitive to insulin because the HK is already bound to the mitochondria is one of the primary propositions of the HK-mitochondrial acceptor theory.[1]

The insulin-like family of molecules is large and diverse. As for the cause of the binding of HK to mitochondria in the process of transition from rest to proliferation, one may speculate that there may be a number of insulin-like proteins whose role is to bind HK to mitochondria in different compartments of the cell, depending on where there is need for energy. At the time of proliferation, this site may be adjacent to the enzymes involved in the pathways needed for cell division. One could envisage that in the course of

development, various insulin-like proteins, which all have domains for the binding of hexokinase, and possibly for porins or other molecules on the mitochondrial membrane, are expressed, targetted to different locations in the cell, and then turned off at the appropriate time. One may speculate that the process of transformation must entail an uncontrolled upregulation of expression of such an intracellular protein to cause the binding of HK to mitochondria, hence stimulate the Kreb cycle of specific mitochondria, and thus provide the dividing cell with accelerated protein synthesis and ATP delivery to sites of nucleotide, protein, and fat synthesis. This principle of "super"-compartmentation in cancer cells is an extension of the compartmentation principle set forth for the role of insulin.[3,48]

Mitochondria of many carcinoma cells are known to have an increased electrical potential across their inner mitochondrial membrane.[49] There is a proto-oncogene product, Bcl-2, which is an integral membrane protein with a molecular mass of approximately 26 kDa, that functions as a suppressor of programmed cell death. Bcl-2 has also been localized to the endoplasmic reticulum, to the nuclear membrane, in addition to the outer mitochondrial membrane.[50] It has been recently noted that Bcl-2 is targeted to the mitochondrial outer membrane by a COOH-terminal signal anchor sequence, and the insertion of Bcl-2 into the mitochondrial outer membrane is mechanistically different from its association with microsmes.[51] Furthermore, it has been noted that the expression of Bcl-2 is highly correlated with cellular energy status in their inverse relationship to sensitivity for glucocorticoid-induced apoptosis.[52] The mechanism by which Bcl-2 overexpression suppresses apoptosis is not clear. It is known that L929 cells overexpressing Bcl-2 have an increased mitochondrial membrane potential and are protected against apoptotic cell killing.[53] The location of Bcl-2 on the mitochondrial membrane lends support to the possibility that this protein may be somehow involved in the mitochondrial energy metabolism. Another pathway also on the cell membrane and deeply involved with the mitochondrial energy production is the aforementioned HK–porin binding. Could Bcl-2 possibly be involved in promoting the binding of HK to the porin by insulin-like proteins? Research in this arena of the role of mitochondria in apoptosis is a fascinating field under major study.

In this article evidence is presented to explain the role of HK–mitochondrial binding in proliferation by using a model developed in the 1960's for the mechanism of insulin action.[1–3] The use of this model enables one to understand the reason underlying the high rates of aerobic glycolysis in cancer cells, and elucidates the Crabtree effect. We propose that HK binding to mitochondria actually lies at the core of proliferative metabolism by providing both the energy and protein requirements of the rapidly dividing cell. We also propose that there are insulin-like proteins expressed intracellularly whose misregulation can lead to proliferation or apoptosis. The goal of this article was to bring more attention to the critical role of HK-Mitochondrial binding and the compartmentation it represents in cancer metabolism.

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