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Hexokinase-mitochondrial interaction in cardiac tissue: implications for cardiac glucose uptake, the ^{18}FDG lumped constant and cardiac protection

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Abstract The hexokinases are fundamental regulators of cardiac glucose uptake; by phosphorylating free intracellular glucose, they maintain the concentration gradient driving myocardial extraction of glucose from the bloodstream. Hexokinases are highly regulated proteins, subject to activation by insulin, hypoxia or ischaemia, and inhibition by their enzymatic product glucose-6-phosphate. *In vitro* and in many non-cardiac cell types, hexokinases have been shown to bind to the mitochondria, both increasing their phosphorylative capacity, and having a putative role in the anti-apoptotic function of protein kinase B (PKB)/Akt. Whether hexokinase-mitochondrial interaction is a dynamic and responsive process in the heart has been difficult to prove, but there is growing evidence that this association does indeed increase in response to insulin stimulation or ischaemia. In this review I discuss the relevance of hexokinase-mitochondrial interaction to cardiac glycolytic control, our interpretation of ^{18}FDG cardiac PET scans, and its possible role in protecting the myocardium from ischaemic injury.

Keywords Hexokinase · Akt · Mitochondria · Lumped constant · Apoptosis

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Introduction: cardiac metabolism of glucose and its PET tracer ^{18}FDG

The significant metabolic load on the heart required to maintain constant beat-to-beat contraction means that it is highly dependent on substrate delivery from the bloodstream. The heart is therefore omnivorous, capable of metabolising a wide range of energy substrates, including fatty acids, glucose, lactate, ketone bodies, and amino acids (Stanley et al. 2005). Under normal conditions, the predominant cardiac fuel are fatty acids, contributing to approximately 60–90% of the heart's energy requirements (Kodde et al. 2007). However, under conditions of increased workload, disease or injury, where oxygen availability may be insufficient to maintain β-oxidation, or after a meal, where blood glucose levels are high, glycolysis assumes a much greater role in maintaining cardiac contractility and viability. We have previously shown, in isolated perfused rat hearts, that cardiac glucose uptake can increase up to 20-fold during low flow ischaemia (Southworth and Garlick 2003).

An increasingly widespread tool for non-invasively quantifying myocardial glucose uptake for both diagnostic and experimental purposes is Positron Emission Tomography (PET)(Underwood et al. 2004). Once injected into a patient, the accumulation of the radiolabelled glucose tracer ^{18}fluoro-2-deoxyglucose (^{18}FDG) can be imaged and quantified by a PET scanner, which detects the gamma rays emitted by the annihilation of positrons from the decay of the ^{18}F nucleus. Most commonly used clinically to detect and stage tumours due to their high glycolytic rate, ^{18}FDG-PET is also the gold-standard technique for identifying "hibernating" myocardial tissue. Hibernating myocardium, thought to be present in up to 40% of all patients with heart disease, is hypocontractile, and its identification by

virtue of its increased ^{18}FDG uptake is a useful prognostic biomarker for successful revascularisation (Heusch et al. 2005).

While ^{18}FDG is widely used as a tracer for glucose metabolism, care must be exercised when extrapolating tissue uptake of ^{18}FDG to true uptake of glucose. Glucose is transported into the cell down a concentration gradient via the glucose transporters (GLUTs), and then phosphorylated to glucose-6-phosphate (G-6-P) by the hexokinases (HKs). This phosphorylation is bi-functional; it ensures low intracellular glucose levels, thereby maintaining the concentration gradient necessary for further glucose entry into the cell, and it also renders the incoming glucose polar, and therefore unable to exit the cell via the GLUTs. Glucose is thus trapped intracellularly and available to the cell for further metabolism. Like glucose, ^{18}FDG is transported and phosphorylated by the GLUTs and HKs respectively, but the hexokinase product ^{18}FDG-6-P is not *generally* considered available for further metabolism (Southworth et al. 2003). The accumulation of ^{18}FDG-6-P within cells with high transport and phosphorylation rates forms the basis for using ^{18}FDG as a PET tracer; catabolism of ^{18}FDG-6-P would result in loss of ^{18}F from the cell, and no appreciable image.

While the difference between ^{18}FDG and glucose is its strength in terms of obtaining a PET image, it is also its weakness in terms of interpreting the PET images we obtain. It is well known that the relative affinities of GLUTs and HKs for glucose and ^{18}FDG are different. This difference is currently accounted for by the use of a "lumped constant" to mathematically relate measured ^{18}FDG-6-P accumulation to glucose uptake in tissues. This correction factor, originally derived to relate autoradiographic measurements of ^{14}C-labelled 2-deoxyglucose (2-DG) uptake to glucose uptake in the brain (Sokoloff et al. 1977), assumes that the transport and phosphorylation rates of glucose and its tracers do not change with respect to each other. While this may be a reasonable assumption for many applications, in a dynamic multi-fuel consuming organ like the heart, the lumped "constant" is famously inconstant (Doenst and Taegtmeyer 1998; Ng et al. 1991). We and others have demonstrated significant differences between cardiac glucose uptake and the uptake of its tracers 2-deoxyglucose (2-DG) and ^{18}FDG under a number of conditions, most importantly post-ischaemia (Garlick et al. 1999; Southworth et al. 2002), where glucose uptake continues or increases, but the accumulation of its tracers do not (Doenst and Taegtmeyer 1998; Liedtke et al. 1992). At best, this seriously limits the accuracy of our measurements of glucose uptake in the experimental setting; at worst, it has the potential to give rise to false negatives in the delineation of potentially salvageable myocardium by ^{18}FDG-PET scanning (Doenst and Taegtmeyer 1998; Knuuti et al. 1993).

We are therefore left with these questions, which form the focus of work in our laboratory, and the remainder of this review. Why does ^{18}FDG not accurately track myocardial glucose uptake after ischaemia (and can we do anything about it?). How does the heart prodigiously increase its ^{18}FDG (and presumably glucose) uptake 20-fold during low-flow ischaemia (and can this be exploited to provide cardiac protection?). We believe that these questions can be answered by better understanding of the regulation and behaviour of HK.

Glucose uptake vs. glucose phosphorylation—which is key?

While the GLUTs have an obvious regulatory role in myocardial glucose and ^{18}FDG uptake, their importance relative to phosphorylation in governing net uptake rate, particularly under conditions of insulin stimulation, high workload or cardiac stress, is difficult to ascertain. Cardiac-specific knockout of GLUT 4 has predictable negative effects on cardiac glucose uptake under basal conditions, and limits recovery post-reperfusion (Tian and Abel 2001), and numerous studies have demonstrated externalisation of GLUT 4 in response to insulin, hypoxia, ischaemia and exercise (Davey et al. 2007; Mueckler 1994; Russell et al. 1999). However, translocation of GLUT 4 to cardiac membranes after ischaemia, or in response to increased extracellular lactate, is not always reflected by increased ^{18}FDG uptake (Garlick et al. 1999; Southworth et al. 2002; Zaha et al. 2005). Since the glucose transporters have a lower K_m for both 2-DG and ^{18}FDG than they do for glucose (Crane et al. 1983), it would be expected that ^{18}FDG uptake would surpass glucose uptake when GLUT 4 externalises; the reverse, however, is observed.

It is established that under conditions of hypoxia, ischaemia and insulin stimulation, glucose phosphorylation by HK is the rate-limiting step, despite increased glucose transport (Morgan et al. 1961). In isolated myocytes, insulin stimulation can achieve intracellular glucose concentrations 10 times higher than the K_m for HK, indicating its saturation (Manchester et al. 1994). While GLUT 4 over-expression has been shown to increase basal glucose uptake in cardiac tissue, this did not translate to an increased capacity for glycolysis in response to insulin, suggesting that glucose transport was not a limiting factor in insulin response (Belke et al. 2001). GLUT 4 over-expression and knockout respectively increase and reduce basal rates of cardiac glucose uptake, while partial knockout of HKII has no effect on basal glucose uptake. However, under conditions of increased glucose demand such as exercise, or insulin stimulation, the impact of HK II deletion on glucose uptake becomes apparent (Fueger et al. 2003; Fueger et al. 2007).

Conversely, over-expression of HKII in skeletal muscle has no effect on basal 2DG uptake rates, but results in a significant capacity to increase tracer uptake during exercise (Halseth et al. 1999). In terms of stress, then, it seems that glucose phosphorylation is more important than glucose transport. This is particularly important when we consider the characterisation of the diseased heart (and its imaging by PET).

Does Hexokinase Translocate to the Mitochondria in cardiac tissue?

It has long been known that cardiac HK associates with mitochondria (Abraham et al. 1964; Borrebaek and Haviken 1985), and numerous studies have demonstrated the increased phosphorylative capacity of HK when it associates with isolated mitochondria *in vitro*, as I shall later discuss. However, whether HK was capable of responsively migrating to the mitochondria in the intact heart has until recently been a matter of debate, due mainly to practical difficulties in assessing hexokinase activity in cardiac tissues. Studying HK redistribution in tissue homogenates by virtue of a shift in enzyme activities from soluble to particulate fractions is difficult to interpret since homogenisation is likely to affect HK-mitochondrial affinity, and resultant measured HK activity (Lynch et al. 1991). Furthermore the fact that HK migration in the heart seems to be heavily modified by the presence of alternate energy substrates seems to have caused some confusion (Doenst et al. 1998; Russell et al. 1992). However, since HK had been shown to migrate to the mitochondria in other tissues (Arora and Pedersen 1988; Gray et al. 1994; Vogt et al. 1998), it seemed unlikely that HK-mitochondrial interaction in the heart should be uniquely different. We therefore employed immunogold labelling and electron microscopy to directly visualise HK biodistribution, thereby circumventing the difficulties in assessing HK redistribution inferred from changes in activity in cell homogenate fractions. Using this technique we observed significant migration of both cardiac HK isoforms to the mitochondria within 30 minutes of either insulin stimulation or ischaemia, and that translocation was significantly limited by the presence of oleate as an alternate energy source (Southworth et al. 2007).

What are the metabolic benefits of HK-mitochondrial interaction?

Hexokinase-mitochondrial binding is mediated by the interaction of a conserved hydrophobic sequence at the N-terminal end of both HKI and HKII isoforms with the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane (Anflous-Pharayra et al. 2007; Lindén et al. 1982; Sui and Wilson 1997), as recently reviewed in this journal (Pastorino and Hoek 2008). This interaction has been proposed to have a number of functions: while both isoforms can be inhibited by G-6-P at their respective C-terminal active sites through competition with ATP, both isoforms are also strongly inhibited allosterically by G-6-P binding in their N-terminal halves (Ardehali et al. 1996; Sebastian et al. 1999). By binding to the mitochondria, HK's vulnerability to product inhibition by G-6-P is markedly decreased, such that glucose phosphorylation may continue despite intracellular G-6-P concentrations many times in excess of its K_i (Ardehali et al. 1999; Arora et al. 1993; Liu et al. 1999). To prevent "runaway glycolysis", G-6-P is able to displace HK from mitochondria, thereby eliciting a double inhibitory effect, by both inhibiting HK glucose phosphorylation directly, as well as increasing its susceptibility to that inhibition by displacing it from the mitochondria (Liu et al. 1999). Interestingly, it has been shown that while inorganic phosphate (P_i) promotes G-6-P dissociation of HKII, it antagonises the dissociation of HKI. Since increases in intracellular P_i and low levels of G-6-P are associated with increased rates of glycolysis, this would suggest a differential glycolytic feedback mechanism on the respective isoforms. In turn, it has been suggested (Wilson 2003) that HKI may have a primarily catabolic role, while HKII's role may be more anabolic, providing glucose for glycogen synthesis and pentose phosphate pathway intermediates (a process normally directed by insulin). Our finding that HKII translocation is significantly more responsive to insulin than HKI (Southworth et al. 2007) is consistent with this assertion.

While mitochondrial-bound HK is capable of utilising cytosolic ATP, by forming a functional interaction with VDAC and the adenine nucleotide translocator (ANT) complex, it has been shown that HK gains preferential access to ATP from the intra-mitochondrial space (Arora and Pedersen 1988; BeltrandelRio and Wilson 1992). While not changing the K_m for glucose, this association does appear to lower the apparent K_m for ATP (de Cerqueira Cesar and Wilson 1998). Through HK-mitochondrial interaction, glucose uptake can thus be closely coupled with ATP generated as a result of oxidative phosphorylation, thereby avoiding the excessive production of lactate (Wilson 2003).

What does HK-mitochondrial interaction mean for ^{18}FDG-PET?

While occurring via different mechanisms, the net effect of either insulin stimulation or ischaemia is a co-operative ramping of both transport and phosphorylation to maximise

glucose uptake. While the K_m of unbound HKI and HKII for ^{18}FDG are 63 and 174 μM respectively, (compared to 40 and 130 μM for glucose)(Muzi et al. 2001), transport rather than phosphorylation is rate-limiting under basal conditions, and glucose tracer uptake therefore reflects (and probably overestimates) glucose uptake. However, at the onset of ischaemia or insulin stimulation, HK phosphorylation becomes rate-limiting. Even under basal conditions in the cytosol, both HK isoforms already preferentially phosphorylate glucose over its tracers; the responsive binding of HK to the mitochondria during ischaemia or insulin exacerbates this, causing a further 8.5-fold increase in its K_m for glucose tracer compared to glucose (Russell et al. 1992). While it is not yet known whether this increase is isoform-specific (and considering their proposed respective roles it is likely (Wilson 2003)), the different affinities, sensitivities to ischaemia and fatty acid modulation make it apparent that differential translocation of the two HK isoforms to the mitochondria has a significant, variable and currently unpredictable effect on the lumped constant. This in turn has serious implications for the reliability of ^{18}FDG-6-P accumulation as a measure of glucose metabolism in the ischaemic heart.

Mechanisms of HK-mitochondrial interaction: a link to apoptosis?

Much attention is currently focused on the potential benefits of HK-mitochondrial association which are not (directly) linked to glucose metabolism. Activation of the serine/threonine kinase Akt (or protein kinase B) is thought to elicit cardiac protection through a variety of mechanisms, some of which promote glucose uptake and/or inhibit apoptosis (Birnbaum 2004; Jonassen et al. 2001). The mechanism underlying apoptosis is currently controversial, as is the identity and structure of the mitochondrial permeability pore itself (Desagher and Martinou 2000; Juhaszova et al. 2008; Newmeyer and Ferguson-Miller 2003); dissociation of HK from the outer mitochondrial membrane, however, appears to be a common thread for many of the proposed mechanisms. Whilst comparing and contrasting the respective mechanisms of apoptosis is outside the scope of this mini-review, I shall briefly summarise some of the evidence which supports HK's putative role in cardiac protection.

As previously discussed, the VDAC/ANT mitochondrial complex allows the exchange of ATP and ADP across the outer mitochondrial membrane. In isolated mitochondria, this ATP/ADP cycle has been shown to maintain the mitochondrial membrane potential, and decrease free radical production. This effect is abolished upon the removal of glucose from the system, or dissociation of HK from the mitochondria by elevated levels of G-6-P (da Silva et al. 2004; Santiago et al. 2008). This suggests that HK activity at the mitochondrion drives and regulates this process; whether this control is sufficient to ameliorate free radical-mediated cell injury or prevent apoptosis *in vivo* is currently unknown. However, HK's phosphorylative activity has been shown to be intimately involved in its cytoprotective role; while DG can act as a phosphate acceptor for hexokinase and maintain this protection, 5-thioglucose, a competitive HK inhibitor, which can not be phosphorylated, abolishes this effect (Gottlob et al. 2001). Thus it seems that maintenance of the ATP/ADP shuttle through the ANT/VDAC complex is key to maintaining mitochondrial function and cell viability, and that this is mediated through HK.

Together, with cyclophilin-D, VDAC and ANT have been proposed to constitute the mitochondrial permeability pore (Beutner et al. 1998). Regulated opening of this pore by the pro-apoptotic proteins Bax and Bak binding to VDAC1 has been suggested to be responsible for cytochrome c release, and the onset of apoptosis. Binding of HK to VDAC antagonises Bax and Bak-mediated apoptosis by competing with the VDAC binding site (Pastorino et al. 2002). Forced removal of HK from the mitochondria, by G-6-P, HKII-specific blocking peptides or jasmonates has been shown to be pro-apoptotic (Gimenez-Cassina et al. 2009; Goldin et al. 2008; Miyamoto et al. 2008). Association of HK with mitochondria is promoted by Akt activation, and Akt inhibition leads to the rapid dissociation of HK from the mitochondria, and apoptosis (Pastorino et al. 2005). Furthermore, the apoptosis caused by forced removal of HK from the mitochondria cannot be salvaged by Akt activation, confirming that it is upstream of HK binding (Majewski et al. 2004).

The exact mechanism by which Akt promotes HK-mitochondrial interaction remains undefined (Pastorino and Hoek 2008). HK has a consensus sequence which suggests it can be phosphorylated by Akt, but phosphorylated HK was found in both mitochondrial and cytosolic fractions, so the significance of this to binding is unclear (Miyamoto et al. 2008). Chronic inhibition of glycogen synthase kinase 3β (GSK3β), a known substrate for Akt (Cross et al. 1995), has been shown to increase HKII binding to the mitochondria, promoting glycolysis and decreasing apoptosis. This may be mediated by alleviation of GSK3β's phosphorylation of VDAC, thereby allowing HK to bind (Gimenez-Cassina et al. 2009; Pastorino et al. 2005).

As Matsui and Rosenzwieg state in their review, there is little point in protecting cells from apoptosis if those that survive are not functional (Matsui and Rosenzweig 2005). Having demonstrated that Akt activation reduced infarct size by 64% after a 30 minute coronary artery ligation, Matsui et al. went on to demonstrate that this protection was glucose dependent in hypoxic isolated myocytes

(Matsui et al. 2001). Disappointingly, in their follow up study, they quantified HKII expression, which was unchanged, but did not investigate its distribution (Matsui et al. 2006). It is more likely in the short term of these experiments, that HK redistribution, rather than HK synthesis, is affording this protection.

It surprising that to date only one study has investigated (and demonstrated) a direct correlation between HK redistribution to the mitochondria and the protective effects of preconditioning in an intact heart (Zuurbier et al. 2005). In light of the numerous mechanisms by which preconditioning may ameliorate apoptosis in the heart, many of which directly or indirectly implicate HK-mitochondrial interaction as an integral part, it is unlikely to be the only study for long. In terms of the lumped constant problem that HK-mitochondrial interaction causes, it seems that we are far from a solution. While greater understanding of the regulation of the HK-mitochondrial relationship will surely come, it is unlikely that we will be able to resolve the multiparametered phenomenon of ^{18}FDG-6-P accumulation into a truly accurate methodology for glucose uptake under all pathophysiological conditions. ^{18}FDG currently enjoys its popularity for many reasons other than its accuracy — availability, ease of synthesis, cost, and the fact that it is a multipurpose PET tracer. However, molecular imaging is currently a burgeoning field of research, and many new tracers are increasingly being used, which are either more accurate (such as ^{11}C-glucose, which is a true glucose tracer (Herrero et al. 2007)), more specific (such as ^{99m}Tc-Annexin V, which binds to apoptotic cell membranes (Murray et al. 2001)), or provide different information (such as ^{64}Cu ATSM, which accumulates in viable but hypoxic tissue (Lewis et al. 2002)). It is likely that many such techniques shall be used in the future to advance our understanding of the relationship between HK and cardiac protection, both in the laboratory, and in the clinic.

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