BJP British Journal of Pharmacology

DOI:10.1111/bph.12363
www.brjpharmacol.org

Themed Issue: Mitochondrial Pharmacology: Energy, Injury & Beyond

REVIEW

Targeting hexokinase II to mitochondria to modulate energy metabolism and reduce ischaemia-reperfusion injury in heart

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Supported by a grant of the Dutch Heart Foundation (NHS2010B011).

Keywords
ischaemia-reperfusion injury; mPTP; cell death; diabetes; glycolysis; mitochondria

Received
1 July 2013
Revised
12 August 2013
Accepted
15 August 2013

Mitochondrially bound hexokinase II (mtHKII) has long been known to confer cancer cells with their resilience against cell death. More recently, mtHKII has emerged as a powerful protector against cardiac cell death. mtHKII protects against ischaemia-reperfusion (IR) injury in skeletal muscle and heart, attenuates cardiac hypertrophy and remodelling, and is one of the major end-effectors through which ischaemic preconditioning protects against myocardial IR injury. Mechanisms of mtHKII cardioprotection against reperfusion injury entail the maintenance of regulated outer mitochondrial membrane (OMM) permeability during ischaemia and reperfusion resulting in stabilization of mitochondrial membrane potential, the prevention of OMM breakage and cytochrome C release, and reduced reactive oxygen species production. Increasing mtHK may also have important metabolic consequences, such as improvement of glucose-induced insulin release, prevention of acidosis through enhanced coupling of glycolysis and glucose oxidation, and inhibition of fatty acid oxidation. Deficiencies in expression and distorted cellular signalling of HKII may contribute to the altered sensitivity of diabetes to cardiac ischaemic diseases. The interaction of HKII with the mitochondrion constitutes a powerful endogenous molecular mechanism to protect against cell death in almost all cell types examined (neurons, tumours, kidney, lung, skeletal muscle, heart). The challenge now is to harness mtHKII in the treatment of infarction, stroke, elective surgery and transplantation. Remote ischaemic preconditioning, metformin administration and miR-155/miR-144 manipulations are potential means of doing just that.

LINKED ARTICLES

This article is part of a themed issue on Mitochondrial Pharmacology: Energy, Injury & Beyond. To view the other articles in this issue visit http://dx.doi.org/10.1111/bph.2014.171.issue-8

Abbreviations

AMPK, AMP-activated PK; ANT, adenine nucleotide transporter; CABG, coronary artery bypass grafting; CypD, cyclophilin D; CytC, cytochrome C; ER, endoplasmic reticulum; G6P, glucose-6-phosphate; HK, hexokinase; HSP90, heat shock protein 90; IMM, inner mitochondrial membrane; IPC, ischaemic preconditioning; IR, ischaemia-reperfusion; miR, micro RNA; mPTP, mitochondrial permeability transition pore; mtHK, mitochondrially bound hexokinase; OMM, outer mitochondrial membrane; PGC-1β, PPAR gamma co-activator 1 beta; RIPC, remote ischaemic preconditioning; ROS, reactive oxygen species; VDAC, voltage-dependent anion channel

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Introduction

The two leading causes of human mortality are cancer and cardiovascular disease. Curiously, the directed goal of therapy against these diseases is diametrically opposed: in cancer, we strive to kill the tumour cells, whereas in heart disease, we strive to protect cardiac cells from being killed. The question arose whether the processes that promote cell survival in cancer cells could potentially be used in promoting survival in the heart, and whether our understanding of the death processes in the heart could be harnessed in the treatment of cancer. The cancer literature has suggested that the resilience of cancer cells against cell death is, at least partly, due to highly elevated levels of hexokinase II (HKII) bound to mitochondria (Pedersen, 2007). Knowing that mitochondrial dysfunction has emerged as a major mediator of cell death in ischaemia-reperfusion (IR) injury of the heart, we considered the possibility that HKII is one of the critical regulators of mitochondrial dysfunction in cardiac IR injury. This consideration was primarily based on the pioneering work by others, demonstrating the pivotal role of HK in mitochondrial function and cell death in non-cardiovascular research fields. Instrumental in these HK pioneering studies were the discoveries of hexokinase (HK) binding to and regulation of mitochondrial voltage-dependent anion channel (VDAC; Fiek *et al.*, 1982; Forte *et al.*, 1987; Brdiczka, 1990; Rostovtseva *et al.*, 2005; Rostovtseva and Bezrukov, 2008) and the regulatory role of HK in mitochondrial pore formation (Nakashima *et al.*, 1986; Marzo *et al.*, 1998).

Various work by us and others have now clearly demonstrated that mitochondrially bound hexokinase II (mtHKII) is indeed a major determinant of infarct size and may constitute one of the end-effectors of ischaemic preconditioning. Mitochondrial hexokinase II (and I) affects mitochondrial membrane potential and reactive oxygen species (ROS) production, regulates mitochondrial permeability transition pore (mPTP) and may also determine the direction of cardiac metabolic flux. This review summarizes current knowledge about the role of mitochondrially bound HK in cardioprotection and cardiac IR injury, adding mtHKII as a new target that may prove useful in the quest to reduce mortality due to cardiovascular disease.

The HK family

The first step in the metabolism of glucose is its phosphorylation upon entrance into the cell. Sufficient and rapid phosphorylation is important to maintain the steep gradient of glucose concentration over the plasma membrane to drive continuous glucose uptake through the GLUT transporters, as well as to render glucose polar, and therefore incapable of exiting the cell. Glucose phosphorylation is catalysed by the enzyme HK, of which four isozymes are present within mammalian tissue. HKI, II and III are isozymes of 100 kDa, displaying high affinity for glucose ($K_m \leq 0.3$ mM) and product inhibition for glucose-6-phosphate (G6P) ($K_i \leq 0.1$ mM), whereas HKIV (glucokinase) is 50 kDa in size, has low affinity for glucose (half-saturation at about 8 mM glucose) and does not show product inhibition at physiological levels of G6P (Wilson, 2003). Importantly, HKI and II contain a hydrophobic amino terminal mitochondrial binding motif, which is not present in the HKIII and IV isoforms.

HKI is ubiquitously expressed in almost all mammalian tissues, is largely unresponsive to hormonal and prevailing metabolic conditions, and can be considered more of a housekeeping protein. Surprisingly, despite being a glycolytic enzyme, and with glycolysis mainly being thought of as a cytosolic process, HKI is predominantly associated with mitochondria (Crane and Sols, 1953; Johnson, 1960; Abraham *et al.*, 1964). It is suggested (Wilson, 1995; John *et al.*, 2011) that HKI principally performs a catabolic function, channelling glucose into glycolysis for ATP production. In contrast, HKII is more variably located in either the cytosol or at the mitochondrial outer membrane and is mostly expressed in insulin-sensitive tissue, such as heart, skeletal muscle and adipose tissue. When situated in the cytosol, it directs glucose into glycogen synthesis, while when bound to mitochondria, it primarily directs glucose into glycolysis (John *et al.*, 2011). HKII expression levels and localization are highly regulated by (patho)physiology, hormones and metabolic state (Heikkinen *et al.*, 2000; Wilson, 2003). In contrast to the abundance of HKI and HKII, HKIII shows low expression in most mammalian tissues, being most highly represented in lung, liver and spleen (Heumann *et al.*, 1974; Furuta *et al.*, 1996). Finally, HKIV is traditionally regarded as a glucose-sensing enzyme (although other HK isoforms also display glucose-sensing properties), associated with regulating insulin release by pancreatic beta cell. This glucokinase is mainly expressed in the liver and pancreas, but can also be found in certain parts of the brain and gut (Postic *et al.*, 2001).

Mitochondrial HK in cancer

As early as 1924, the pioneering work of the Nobel prize winner Otto Warburg (Warburg *et al.*, 1924) associated malignant, aggressive tumour growth with increased rates of aerobic glycolysis and increased lactate production, a signature now known as the Warburg effect (Pedersen, 2007). It took another 50 years to demonstrate mitochondrial HKII as a key molecular governor of this increased glycolysis (Bustamante and Pedersen, 1977; Bustamante *et al.*, 1981), with the expression of HKII (sometimes HKI) being increased often more than 100-fold. Activation of the PI3K/Akt pathway, one of the most frequently mutated pathways in cancer (Shaw and Cantley, 2006), and activation of PKA and PKC pathways, also commonly seen in cancers, may explain this increased HKII expression. DNA demethylation and HKII gene amplification has also been suggested to play a role (Mathupala *et al.*, 2009), as has the increased expression of hypoxia-inducible factor HIF1α (Semenza, 2003; Keith *et al.*, 2012). Although Warburg originally hypothesized that the increased reliance of tumours on glycolysis was due to impairments in mitochondrial function, it is now known that this is not the case. Mitochondria from tumours can still have normal oxidative phosphorylation with intact ATP synthetic capacity; however, they are often reprogrammed towards biosynthetic pathways supporting tumour proliferation, such that glucose and glutamine become important substrates

feeding rewired anabolic pathways (Ward and Thompson, 2012). Thus, the increased HKII expression and its binding to mitochondria facilitates not only increased aerobic glycolysis and lactate production (John *et al.*, 2011) but also the channelling of glycolytic substrates into biosynthetic pathways for which mitochondria play a crucial role. The increase in the proportion of HKII that is bound to mitochondria also provides the cancer cell with resilience to cell death. The exact mechanism of this protection has not yet been elucidated, but it is known that glucose is necessary in order for mtHKII to inhibit apoptosis, indicating that this is an active process requiring glucose phosphorylation (Gottlob *et al.*, 2001). Targeting the binding between HKII and mitochondria is currently actively pursued as a possible treatment against aggressive proliferative tumours. Such targeting may be achieved with 3-bromopyruvate (Mathupala *et al.*, 2009), methyl jasmonate (Goldin *et al.*, 2008), dichloroacetate (Michelakis *et al.*, 2010), the antifungal compounds clotrimazole and bifonazole (Penso and Beitner, 1998), and some traditional Chinese medicinal plants (Wei *et al.*, 2013). However, such treatment comes with a price. Our recent data (Smeele *et al.*, 2011a), showing the high sensitivity of heart towards disruption of mtHKII binding and the immediate development of cardiac cell death, are a direct warning against any such treatment being a global, whole-body treatment. A nice example of this phenomenon is the use of anthracyclines in cancer chemotherapy, which, while very effective in cancer treatment, have to be very carefully titrated because of their severe cardiotoxicity. It has been suggested that this cardiotoxicity may be mediated in part by mito-HK dissociation via inhibition of Akt signalling (Pastorino *et al.*, 2005). Thus, it is of paramount importance that these drugs target the cancer cell through localized delivery to the tumour (Ko *et al.*, 2012) or using compounds that are only taken up by the cancer cell through selective cancer-expressed transporters (Birsoy *et al.*, 2013).

Mitochondrial HK and protection in non-cardiac tissues

Although the primary focus of this review is on the heart, mitochondrial HK has been shown to protect against stressors in several other organs and tissues. We demonstrated that decreased (mitochondrial) HKII increased IR injury in skeletal muscle (Smeele *et al.*, 2010; 2012). In fact, skeletal muscle IR injury was very sensitive to reductions in HKII (50% HKII reduction increased IR-induced cell necrosis from 36 to 76%; Smeele *et al.*, 2012), probably because HKII is the major HK isoform in skeletal muscle. Bryson *et al.* (2002) and Gall *et al.* (2011) showed that increased HK activity protects kidney epithelial cells against oxidant injury, whereas Ahmad *et al.* (2002) demonstrated that HKII protected human lung epithelial cells against hyperoxia and oxidative stress. Finally, mtHKII protects against neurodegeneration in models of Parkinson's disease (Gimenez-Cassina *et al.*, 2009; Corona *et al.*, 2010). Therefore, it seems that the interaction of HK with mitochondria constitute an endogenous cellular protective mechanism against cell death that is operative in many tissues and organs.

Mitochondrial HK and metabolism

Early observations (Bessmann and Geiger, 1980; Wilson, 1995), later supported by various other researchers, demonstrated that mitochondrially bound HK preferentially uses mitochondrially produced ATP (in contrast to cytosolic ATP), with direct channelling of the produced ADP back into the mitochondria. Thus, one major metabolic consequence of HK translocation to the mitochondria is that the ATP sensitivity of glucose phosphorylation is shifted from a cytosolic to a mitochondrial ATP source. Surprisingly, however, it remains unknown what the metabolic consequences of this translocation *per se* are in terms of oxygen consumption and energy substrate selection in intact organs/tissues. The role of mtHK in cell death however is better understood. Several seminal studies have now clearly shown the association between mtHK disruption and cell death, induced by stimuli such as H_2O_2 and/or UV irradiation in cellular studies (Gottlob *et al.*, 2001; Ahmad *et al.*, 2002; Bryson *et al.*, 2002; Pastorino *et al.*, 2002) or IR in intact organs and tissues (Smeele *et al.*, 2011a, 2012; Pasdois *et al.*, 2013). There are indications that enhancing mtHKII may increase (glucose-mediated) oxidative phosphorylation and therefore overall energy production in permeabilized human fibres from dilated atria (Roosimaa *et al.*, 2013). These findings are in line with older literature showing that HK can display high control strength over respiration in isolated mitochondria (Groen *et al.*, 1982). Interestingly, it has also been suggested that an increased translocation of HKI to mitochondria increases the glucose sensitivity of the pancreatic beta cell to release insulin (Rabuazzo *et al.*, 1997). Whether mtHK may affect cardiac (and skeletal muscle) insulin sensitivity is unknown. Clearly, more work needs to be done to answer these important questions as to how mtHKII affects metabolism of the intact heart.

Older literature indicates that mtHK not only affects glucose metabolism but may also regulate cardiac fatty acid oxidation. Mitochondrial HK inhibits palmitoyl-CoA synthetase through competition for ATP and thereby inhibiting palmitate activation at the outer mitochondrial membrane (OMM) in isolated mitochondria (De Jong and Hülsmann, 1970). The reported decrease in mtHK associated with diabetes (Katzen *et al.*, 1970) may therefore be a contributing factor to the often increased fatty acid metabolism in diabetic hearts. The reciprocal relationship was demonstrated by Southworth *et al.* who showed that perfusion of isolated hearts with fatty acids dislodged HKI and HKII from mitochondria (Southworth *et al.*, 2007). Thus, mtHK seems to be an ideal localization hub for the well-known competition between glucose and fatty acid metabolism and the regulation thereof.

Mitochondrial HK in cardiovascular diseases (Figure 1)

HKI and HKII are both present in the heart. In adult mouse heart, HKI and HKII contribute approximately equally to total cardiac HK activity (Smeele *et al.*, 2011b), depending on the age and the nutritional/pathophysiological condition of the animal. Few data are available for human heart, except for

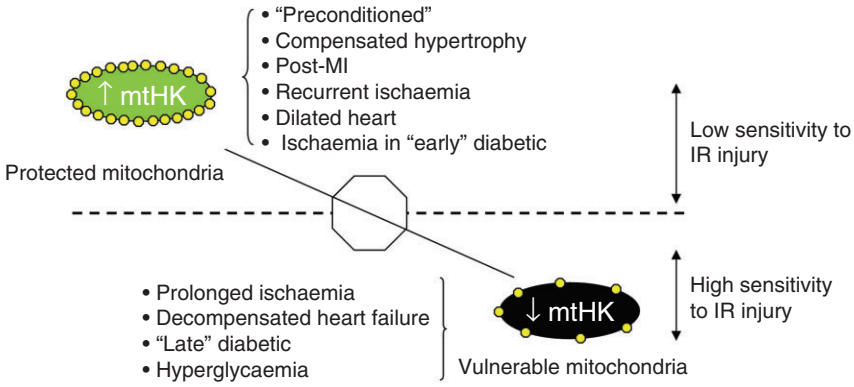

Figure 1

Schematic representation of cardiac disease states with low or high sensitivity to IR injury, which is associated with protected mitochondria (high mtHK) or vulnerable mitochondria (low mtHK) respectively.

a recent report by Roosimaa *et al*. (2013), which indicates that in non-dilated human atrial tissue, HKI is the most abundant isoform. In mouse and human skeletal muscle (Mandarino *et al*., 1995; Smeele *et al*., 2011b), however, HKII accounts for >80% of total HK activity.

Ischaemic preconditioning (IPC) protection against I/R injury through increased mtHKII

The discovery that short, non-lethal periods of ischaemia activate an endogenous cardiac protection programme against long, lethal, periods of ischaemia led to a highly intensified research effort to elucidate the cellular mechanisms. This phenomenon was called IPC (Murry *et al*., 1986). Subsequent research demonstrated that IPC cellular signalling converged on the mitochondrion and was associated with alterations in glycolysis (Murry *et al*., 1986; Murphy and Steenbergen, 2008). Work from our laboratory at that time also noticed this interaction between glycolysis and mitochondrial function following non-lethal periods of ischaemia. We demonstrated that the activation of mitochondrial oxygen consumption, due to an instantaneously increased cardiac workload (Zuurbier and van Beek, 1997; Van Beek *et al*., 1998), was slowed following non-lethal periods of ischaemia. However, no such slowing was observed when glycolysis was bypassed using high concentrations of pyruvate or lactate (Zuurbier and Ince, 2002). In other words, the changes observed in mitochondrial function following non-lethal ischaemia were precipitated through changes evoked in glycolysis. After having established that the alterations in glycolysis were not due to alterations in the pentose phosphate pathway (Zuurbier *et al*., 2004), translocation of HK with non-lethal periods of ischaemia became a likely candidate (Zuurbier *et al*., 2009). Finally, we demonstrated that reversible ischaemia such as IPC induced a translocation of the glycolytic enzyme HK to the mitochondria (Zuurbier *et al*., 2005; Gürel *et al*., 2009). This leads us to the hypothesis that IPC could, at least partly, be attributed to increased HKII trafficking to the mitochondria (Zuurbier *et al*., 2009). This hypothesis was confirmed by subsequent studies showing loss of IPC protective effects with a peptide blocking mito-HKII binding (Smeele *et al*., 2011a) and increased IR injury with partial deletion of the HKII gene in both heart and skeletal muscle (Smeele *et al*., 2010; 2012; Wu *et al*., 2011). This work was recently confirmed by findings in Halestrap's laboratory, showing a close correlation between cardiac infarct size and the extent of mitochondrial HKII dissociation (Pasdois *et al*., 2013).

IR, post-myocardial infarction, regeneration

HK expression and cellular localization changes dramatically during and after periods of ischaemia. During prolonged cardiac ischaemia, there is an increase in cytosolic HK activity (Correa *et al*., 2008) that can be explained by solubilization of HKII (but not HKI) from the mitochondria (Gürel *et al*., 2009; Pasdois *et al*., 2011). The mechanism of HK detachment from the mitochondria during ischaemia is, at least partly, related to acidification and increases in G6P (Pasdois *et al*., 2013). The endogenous adaptation following ischaemia in cardiac tissue is associated with increases (~30–70%) in total and mitochondrial HK (McFalls *et al*., 2002; Miyamoto *et al*., 2010; Wu *et al*., 2011; Yeih *et al*., 2011). Large increases (200–300%) in HK activity were also observed in regenerated skeletal muscle 2 weeks after the IR insult (Smeele *et al*., 2012). It thus seems that following an ischaemic episode, there is increased HK expression, presumably enhancing biosynthetic pathways for regeneration and growth and to offer protection against recurrent episodes of ischaemia.

Hypertrophy and heart failure

During pressure overload-induced cardiac hypertrophy, mtHKII and total HKII protein content have been shown to increase (Riehle *et al*., 2011; Wu *et al*., 2012). Increased HKII expression was also recently reported in dilated human atria (Roosimaa *et al*., 2013). Genetic reductions in HKII resulted in exaggerated cardiac hypertrophy in a pressure-overload model (Wu *et al*., 2012). These data suggest that the increased HKII expression in pressure-overloaded hearts is an adaptive response, possibly attenuating hypertrophy through diminishing oxidative stress (Wu *et al*., 2012). Therapeutic enhancement of tissue HKII during this hypertrophic stage may therefore be a potentially beneficial approach. Interestingly, while mild pressure-overload results in a relatively

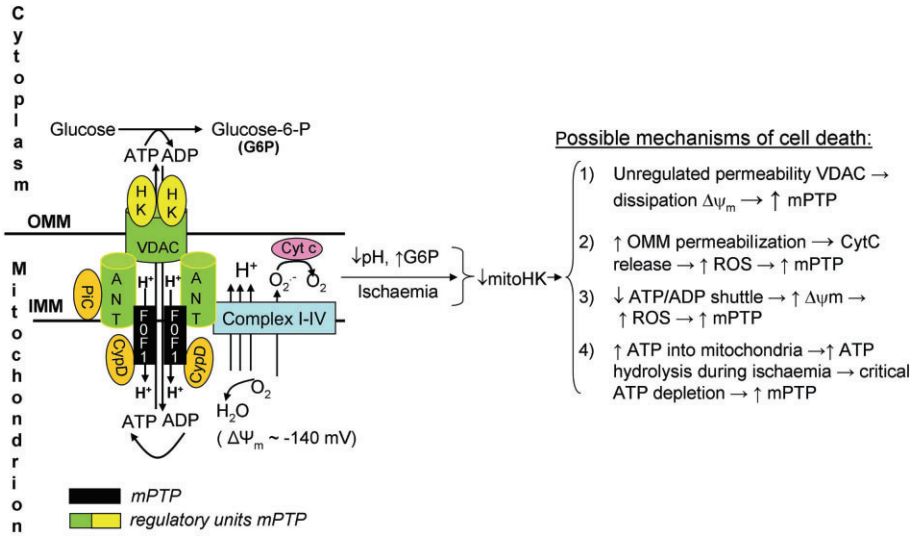

Figure 2

Schematic drawing of the mitochondrial permeability transition pore complex dictating cell death through decreases in mtHK induced by ischaemia-reperfusion. The most likely mechanisms (1–4) through which disruption of hexokinase II-mitochondrial binding may cause cell death are displayed (see text for further discussion).

compensated hypertrophy associated with increases in HKII, the same procedure in animals deficient for PPAR gamma co-activator 1 beta (PGC-1β) resulted in decreased HKII levels, decompensated hypertrophy and evidence of increased oxidative stress (Riehle *et al.*, 2011). These data suggest that PGC-1β, a regulator of mitochondrial biogenesis and genes encoding for mitochondrial metabolism, is needed to maintain cardiac function following pressure overload by possibly preserving HKII expression and preventing oxidative stress. Cardiac HKII has also been shown to be severely reduced in a pacing-induced heart failure model in pigs (Lionetti *et al.*, 2009). While these data suggest that the development of heart failure is associated with decreases in HKII, further studies are warranted to examine whether the reduced HKII protein levels are cause or consequence of cardiac failure.

Hyperglycaemia and diabetes

Hyperglycaemia in the clinical condition is currently viewed as an important risk factor for poor clinical outcome. In addition, the presence of hyperglycaemia in the pre-diabetic state is a strong predictor of developing diabetic disease. Acute hyperglycaemia has been shown to induce the detachment of HK from mitochondria (Da-Silva *et al.*, 2004; Pasdois *et al.*, 2013), which may underlie its pathology. During the development of diabetes, there is a shift in cardiac metabolism away from glucose metabolism towards fatty acid metabolism. This shift is generally associated with significant decreases in cardiac and muscle HKII protein content without alterations in HKI protein content (Katzen *et al.*, 1970; Vestergaard *et al.*, 1995). The diabetic heart displays an altered response to IR and IPC, with short-term diabetes frequently offering protection against IR and attenuated IPC potential, and long-term diabetes resulting in worsened outcome after IR and loss of IPC protective effects (Miki *et al.*, 2012). It is possible that changes in cardiac HK contribute to such an altered response of diabetic heart to IR and IPC. Indeed, we have demonstrated that the increased protection against IR and the attenuation of IPC in the short-term type I diabetic heart are associated with altered mitochondrial HK binding characteristics (Gürel *et al.*, 2013). These data suggest that the known association between diabetes mellitus and ischaemic cardiovascular disease may partly stem from alterations at the level of cardiac HKII expression. More work is needed to fully explore the role of HKII in diabetic cardiomyopathy.

Mechanisms of mtHKII-induced protection against IR injury (Figure 2)

There is currently no consensus concerning the exact mechanism of mtHK protection against IR injury. Below, we restrict our discussion to those four mechanisms for which experimental evidence can be found. The overarching action of mtHK is its regulatory role in the homeostatic crosstalk between the mitochondria and the cell. This crosstalk is set by the degree of permeability of the OMM and the IMM to extramitochondrial metabolites.

The permeability of the OMM is determined by VDAC, the mitochondrial binding partner of HK. HK, at least partly, regulates the permeability of VDAC for many important metabolites, such as ATP, ADP and NADH (Rostovtseva *et al.*, 2005). The permeability barrier of the IMM to protons and ions is essential to ATP synthesis by mitochondria; loss of this permeability barrier prevents the generation of an electrochemical gradient (mitochondrial potential) that drives ATP synthesis through the F_0F_1ATPase. This loss of the IMM permeability barrier, as reflected by the opening of a non-specific pore in the inner mitochondrial membrane (IMM), the so-called mPTP, is currently considered the final event

causing IR injury and irreversible cell death (Halestrap, 2009). The molecular identity of the mPTP was recently suggested to consist of dimers of the F_0F_1ATPase localized on the IMM (Giorgio *et al.*, 2013), with HK, VDAC, adenine nucleotide transporter (ANT) and cyclophilin D (CypD) as important regulators of the mPTP (see also Figure 2). It is through this mPTP complex that HK mediates its decisive role in IR injury and cardioprotection. It should be noted that there may also be a role for the mitochondrial phosphate carrier (PiC) as important regulator of the mPTP (Leung *et al.*, 2008). The reader is directed to literature that discusses this component in detail (Leung and Halestrap, 2008; Halestrap, 2009). For this review, we focus primarily on how HK may affect the mPTP.

Prevention of conformational change in molecular mPTP regulation complex to stabilize mitochondrial membrane potential

It has been shown that mitochondrial depolarization occurs during cardiac ischaemia and may be an early sign of irreversible injury (Lyon *et al.*, 2010). Mitochondrial depolarization during ischaemia and early reperfusion may cause direct mPTP opening (Bernardi, 1992), and there is evidence that mtHKII may prevent such depolarization. We have recently demonstrated that acute HKII detachment from mitochondria in the beating heart *per se* (without any concomitant stress signal), using medium-to-high dosage (≥2.5 μM) of an HKII dislodging peptide, acutely depolarized mitochondria and induced cell death, an effect that could not be explained by vascular obstruction or ensuing ischaemia in the intact organ (Smeele *et al.*, 2011a; Nederlof *et al.*, 2013). In these conditions of normoxic perfusion with a pH > 7 buffer, this depolarization can cause immediate mPTP opening and cell death (Bernardi, 1992). Previously, Chiara *et al.* (2008) demonstrated that the sensitivity of isolated cardiomyocytes to ROS-induced mPTP opening was much increased with low concentrations (<1 μM) of the HKII dislodging peptide. They hypothesized that detachment of HKII induced a conformational change in the molecular complex connecting the OMM with the IMM and the mPTP (Chiara *et al.*, 2008). We would suggest that the HKII dislodging peptide fully opens VDAC, either due to loss of HK or other VDAC-regulating proteins, such as tubulin (Sheldon *et al.*, 2013). VDAC is the most abundant protein of the OMM and is responsible for the transport of ADP/ATP and other metabolites (e.g. NADH, Ca^{2+}) across the OMM (Colombini, 2004; Rostovtseva *et al.*, 2005; Rostovtseva and Bezrukov, 2008). VDAC can change between an open and 'closed' state, which is almost impermeable to ADP and ATP, and may thereby regulate mitochondrial respiration. The binding of several cytosolic proteins (tubulin, tBid, Bcl-x_L, HK) with VDAC, in combination with OMM lipid composition and transmembrane potential, regulate VDAC conductance. It was recently demonstrated that dimeric tubulin, known to interact with mitochondria *in vivo*, induced voltage-sensitive closure of VDAC, reducing ADP availability and thereby mitochondrial respiration (Rostovtseva *et al.*, 2008). Preliminary data (Sheldon *et al.*, 2013) now show that the HKII dislodging peptide disrupts HKII and tubulin binding to VDAC in planar lipid bilayers, resulting in unregulated permeability of VDAC that may ultimately results in large ADP influx, decreasing the mitochondrial membrane potential to a critical level of spontaneous mPTP opening.

Stabilization of mitochondrial contact sites during ischaemia preventing cytochrome C (CytC) release

mtHKII may alternatively prevent OMM rupture and/or permeabilization by stabilization of mitochondrial contact sites. HK is preferentially bound to mitochondria at locations where the IMM comes closest to the OMM (Brdiczka *et al.*, 2006). These contact sites contain large protein complexes consisting of VDAC, ANT, CytC, benzodiazepine receptor, CypD, cardiolipin, HK and creatine kinase. In reconstituted vesicles, disruption of HK from these protein complexes increases their permeability, which could be interpreted as analogous to increased permeability of the OMM and/or mPTP (Beutner *et al.*, 1998). It has been suggested that during ischaemia, progressive acidosis and G6P accumulation dislodge HK from mitochondria (Pasdois *et al.*, 2013). Such decrease in mtHK, together with increased Ca^{2+}, disrupts the contact sides resulting in an increased permeability of the OMM for CytC release. Knowing that oxidized CytC is an important antioxidant, the loss of CytC will result in increased ROS production at early reperfusion (Pasdois *et al.*, 2011; 2013), finally resulting in mPTP opening and infarction.

Maintaining mitochondrial ADP to inhibit mPTP and reduce ROS

The activity of HK at the mitochondrial surface rapidly returns ADP back to the inner mitochondrial compartment via VDAC and ANT, thereby ensuring high levels of ADP in the vicinity of F_0F_1 ATPase dimers at early reperfusion. This 'ATP/ADP' shuttle has been shown to reduce the mitochondrial membrane potential and limit ROS production (Da-Silva *et al.*, 2004; Santiago *et al.*, 2008; Wu *et al.*, 2012). The reduction in ROS production offered by active mtHK can range from >90% (going from zero to normal mtHK levels in isolated brain mitochondria; Da-Silva *et al.*, 2004) to 70% (with activation of mtHK in isolated rat heart mitochondria; Santiago *et al.*, 2008) and to 20% (with 40% increase in mtHKII in neonate rat cardiomyocytes; Wu *et al.*, 2012).

Inhibition of ATP hydrolysis during ischaemia

Maintaining HKII at the mitochondria during ischaemia may impair cytosolic ATP entrance into the mitochondria (Perevoshchikova *et al.*, 2010) during ischaemia, thereby attenuating mitochondrial hydrolysis of cytosolic ATP (through reversed mode of mitochondrial F_1F_0 ATPsynthase). Prevention of a critical depletion of ATP by the mitochondria during ischaemia can significantly reduce anaerobic glycolysis and lactate accumulation, cardiac contracture and cell death induced by IR interventions (Steenbergen *et al.*, 1990; Jennings, 2013).

Structural obstruction of pro-apoptotic protein binding to mitochondria

It has been proposed, in cellular studies employing oxidant agents, that mtHKII may be cytoprotective through structural

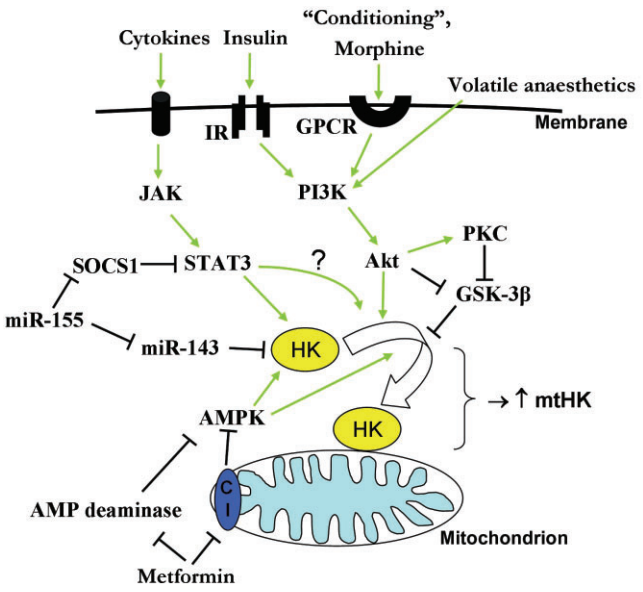

Figure 3

Intracellular signalling pathways showing how different interventions ('conditioning') and compounds such as cytokines, insulin, metformin, miRNA's and anaesthetics may increase HKII expression and/or HKII translocation to mitochondria. IR, insulin receptor; SOCS1, suppressor of cytokine signalling 1; C1, complex I of the electron transport chain.

opposition to the mitochondrial binding of pro-apoptotic proteins such as Bax or Bad (Pastorino *et al.*, 2002). However, we and others have been unable to show a role for Bax in mtHKII-mediated protection against IR injury in isolated hearts (Smeele *et al.*, 2011a; Pasdois *et al.*, 2013) or skeletal muscle (Smeele *et al.*, 2012). The purely structural basis of mtHKII cytoprotection by steric hindrance is also challenged by several studies which demonstrate that glucose must be present in order for HK to offer protection against cell death (Gottlob *et al.*, 2001; Mergenthaler *et al.*, 2012). Furthermore, Majewski *et al.* (2004) demonstrated that the protection offered through HK-mitochondria interaction does not necessitate the presence of Bax and Bak. Thus, it seems that Bax and Bad do not play a significant role in mtHKII protection against IR injury.

Targeting HK to mitochondria (Figure 3)

It is clearly established in numerous cardiovascular disease models and in skeletal muscle IR interventions that the amount of HKII bound to mitochondria is a major determinant of infarct size and/or disease progression (Smeele *et al.*, 2010; 2011a; 2012; Wu *et al.*, 2011; 2012). In fact, the Halestrap laboratory recently reported a strong inverse correlation between the amount of end-ischaemic mtHKII and infarct size in the isolated rat heart subjected to different perfusion condition: tripling of mtHKII reduced infarct size from 65 to 5% (see figure 9, Pasdois *et al.*, 2013). Development of new therapies directed at increasing and/or keeping HKII at the mitochondria therefore seems like an attractive cardioprotective approach. Below we summarize the most promising interventions that have been shown to raise total HK or specifically mtHKII, for exploration of their (clinical) potential as adjunct therapy in settings of ischaemia-reperfusion and possible chronic diseases such as diabetes, cardiac hypertrophy and heart failure.

'Conditioning' of the heart

In 2005, we demonstrated that an IPC stimulus immediately translocates HK to the mitochondria in isolated rat hearts (Zuurbier *et al.*, 2005). Subsequently, we have also shown that IPC also prevents HKII detachment from mitochondria during the irreversible period of ischaemia (Gürel *et al.*, 2009). These studies were later partly confirmed by Pasdois *et al.* (2011; 2013). Such IPC interventions can be performed clinically for leg or arm surgeries, but applicability for the heart is not directly feasible. However, the discovery that the heart can also be protected through remote ischaemic preconditioning (RIPC) or post-conditioning opened an avenue to cardiac application of the 'conditioning' phenomenon. Since the intracellular signalling pathways providing protection with IPC overlap to a large extent with those of RIPC (e.g. Li *et al.*, 2011), it is anticipated that RIPC may also translocate HKII to mitochondria. This information is however lacking in the literature. Our currently active clinical mtHK-RIPC trial (NTR2915, Nederlands Trial Register) of RIPC in coronary artery bypass grafting (CABG) patients examines the relationship between RIPC protection and mtHKII in human atrial tissue and will hopefully provide this much needed information.

Insulin/PI3/Akt/glycogen synthase kinase (GSK)-3β axis

Several studies have demonstrated that activation of this pathway will acutely (<30 min) increase HKII trafficking to the mitochondria (Russell *et al.*, 1992; Pastorino *et al.*, 2005; Zuurbier *et al.*, 2005; Southworth *et al.*, 2007; Miyamoto *et al.*, 2008) and later on increases HKII expression. Juhaszova *et al.* (2004) demonstrated that many cardioprotective agents confer protection through phosphorylation of GSK-3β and consequently inhibition of mPTP. Although many other studies can subsequently be found that have demonstrated cardioprotection with activation of this pathway, none of them have directly examined whether this was due to increased mtHKII (e.g. Hausenloy *et al.*, 2005; Terashima *et al.*, 2010; Ng *et al.*, 2012). A disadvantage of using this pathway to advance mtHKII is of course its pleiotropic character, with unwanted side effects increasing with every step upstream above HKII. For example, insulin may increase HKII translocation to mitochondria, but at the same time may not only result in hypoglycaemia, but also in hypolipidaemia (Zuurbier *et al.*, 2008a). It therefore seems advisable to use more specific treatment for increasing mtHKII.

Anaesthesia

The use of specific anaesthetics for providing protection against IR injury is an attractive scenario in surgeries that necessitate anaesthesia. Pre-clinical studies in healthy, young

animals almost all report protective effects of certain volatile anaesthetics (sevoflurane, isoflurane) and opiates (morphine). These protective anaesthetics also commonly activate the PI3/Akt/GSK-3β axis. However, these protective effects were not always observed in clinical studies employing, for example, CABG procedures (De Hert, 2011). The ambiguity in the clinical scenario potentially results from co-morbidities (diabetes, ageing and hypertrophy) and co-medications (e.g. statins, dexamethasones, opioids, nitroglycerine, β-blockers) that disturb the signalling pathway. Using several different anaesthetic regimens in healthy rats, we were able to demonstrate that the cardioprotective volatile anaesthetics sevoflurane and isoflurane did indeed maintain HK at cardiac mitochondria at a level similar to the non-anaesthetized animal (Zuurbier *et al.*, 2008b). An anaesthetic regimen of propofol–sufentanil–morphine, also often used in the clinical arena, resulted in solubilization of HK from the mitochondria. It can be speculated that the divergent effects of these two clinically most used anaesthetic regimens (volatile anaesthesia vs. fentanyl–propofol anaesthesia) on mtHKII may also explain the dissipating effect of propofol anaesthesia on RIPC protection (Kottenberg *et al.*, 2012). Further studies will be needed to test this hypothesis directly. In conclusion, although certain anaesthetics may indeed offer protection through increases in mtHKII, the many cellular signalling steps that exist between the start of protection induced by an anaesthetic agent to the final subcellular HKII translocation is prone to be disturbed by many clinical and disease factors, thereby decreasing its likeliness as an ideal option offering IR protection under clinical conditions.

Metformin

The glucose-lowering anti-diabetic drug metformin has been shown to be cardioprotective beyond its anti-hyperglycaemic properties. It reduces infarct size in both diabetic and non-diabetic animals (Bhamra *et al.*, 2008; Calvert *et al.*, 2008; Solskov *et al.*, 2008; Whittington *et al.*, 2013). In addition, multiple clinical studies showed reduced cardiovascular mortality in diabetic patients treated with metformin (El Messaoudi *et al.*, 2013). These cardioprotective effects may be caused by increased mtHK activity. Metformin treatment reverses the down-regulation in total HK activity and has been shown to increase HK translocation to the mitochondria in diabetic hearts without adversely affecting normal hearts (Da Silva *et al.*, 2012). Whether metformin will increase (mt)HK in hearts during IR to afford protection remains to be examined, but 2 weeks of metformin treatment has previously been demonstrated to increase HK activity in rat white gastrocnemius muscle (Suwa *et al.*, 2006). This increase in HK might be explained by an increase in Akt phosphorylation after metformin treatment during reperfusion (Bhamra *et al.*, 2008). However, Akt phosphorylation was not observed when metformin was given before ischaemia (Bhamra *et al.*, 2008; Calvert *et al.*, 2008). Alternatively, metformin may increase HK activity through activation of AMP-activated PK (AMPK). Increases in AMPK activity have been associated with increased HK activity in skeletal muscle (Holmes *et al.*, 1999; Dieni and Storey, 2011). Metformin may activate AMPK through increases in AMP resulting from inhibition of complex I (Owen *et al.*, 2000) and/or AMP deaminase (Ouyang *et al.*, 2011; Vytla and Ochs, 2013). Metformin is one of the most long-standing prescribed drugs in diabetes, showing relatively high efficacy. It will be interesting to elucidate whether a common mechanism of this drug entails the translocation of HK to the mitochondria, to identify a cellular mechanisms for its cardioprotective effects.

miR-155/miR-143 therapy

MicroRNAs (miR) have recently been discovered as yet another level of regulation in biological function. miRs are non-protein-coding RNAs of 20–30 nucleotides, which silence gene expression at the post-transcriptional level by targeting the 3′-untranslated region of messenger RNA. The use of miR therapeutics in cardiovascular medicine is only starting to develop, although several studies have already demonstrated the feasibility of such an approach (Thum, 2011). HKII has been shown to be regulated by miR-155 and miR-143 in cancer cells (Jiang *et al.*, 2012; Peschiaroli *et al.*, 2012). Increases in miR-155 induced HKII expression through activation of a HKII transcriptional activator (STAT3) and repression of a negative regulator of HKII, miR-143. Matkovich *et al.* (2013) recently demonstrated that cardiac overexpression of miR-143 was also able to suppress HKII mRNA in the heart with no indirect target regulation. Therapies may therefore be developed which employ synthetic complementary oligonucleotides against miR-143 and/or adeno-associated virus containing miR-155 to specifically increase cardiac HKII and offer protection during IR interventions.

Heat shock proteins

HKII has recently shown to be released from mitochondria in tumour cells during inhibition of mitochondrial heat shock protein 90 (HSP90; Chae *et al.*, 2012). HSP90 has previously been demonstrated to have cardioprotective capacity (Latchman, 2001; Xiang *et al.*, 2010) and the mitochondrial heat shock protein, tumour necrosis factor receptor-associated protein 1 (TRAP1), is known to be present in the heart (Xiang *et al.*, 2010). A therapy directed at increasing mitochondrial HSPs, either through heat treatment or gene therapy, may therefore also have cardioprotective potential by increasing mtHKII.

Detrimental effects of persistent elevated mtHKII in the heart?

The previous sections illustrate the many pro-survival effects of augmenting HKII-mitochondria binding within the heart. This raises the question that if mtHKII is such a powerful protector of the myocardium, why is HKII not permanently bound to mitochondria in the healthy heart? We hypothesize that irreversibly bound mtHK would hamper the natural occurrence of mitochondrial depolarization needed to maintain mitochondria healthy. Since mitochondrial depolarization is a trigger signal for mitochondrial recycling through mitophagy, prevention of depolarization may result in accumulation of dysfunctional mitochondria and ultimately a dysfunctional heart. This is supported by the apparent benefit afforded by dichloroacetate (DCA) in the treatment of pulmonary hypertension, right heart failure (McMurtry *et al.*,

2004), hyperthyroid hypertrophy (Atherton *et al.*, 2011) and cancer (Bonnet *et al.*, 2007; Michelakis *et al.*, 2010). DCA induces mitochondrial depolarization, which may at least partly be explained through disruption of HK-mitochondrial binding (Michelakis *et al.*, 2010). The often observed hyperpolarized mitochondria in cancer cells (Chen, 1988; Bonnet *et al.*, 2007) is consistent with their increased mtHKII as characterized by the Warburg effect. Permanently increasing mtHKII binding would also increase baseline aerobic glycolysis, and subsequently retard cellular signalling and mitochondrial activation, required for rapid responsive change in cardiac workload (Zuurbier and Ince, 2002; Harrison *et al.*, 2003). Such retardation of mitochondrial activation may temporarily impair the free energy of ATP hydrolysis through elevation of cytosolic ADP (Van Beek *et al.*, 1998). Finally, the up-regulated glycolysis caused by permanent association of HK to cardiac mitochondria would presumably switch off fatty acid oxidation through the Randle effect, resulting in a less efficient heart when oxygen is not limiting, and a redirection of fatty acids towards lipid accumulation in the heart. The chronic elevated glucose uptake may also result in an increased accumulation of G6P,, resulting in chronic mammalian target of rapamycin (mTOR) activation concomitant with an activated endoplasmic reticulum (ER) stress response (Sen *et al.*, 2013). Interestingly, lipid accumulation, chronic mTOR activation and ER stress are all biochemical markers of cardiac failure (Shioi *et al.*, 2003; Sharma *et al.*, 2004), a known condition with elevated cardiac HK. It should be realized that the schemes discussed above are purely hypothetical; no evidence currently exists that has demonstrated detrimental effects of excessive mtHKII levels for the heart. However, future work may be devised to provide answers to these important questions of possible detrimental effects of too much mtHKII for the heart.

In conclusion, the association of HKII with mitochondria is a critical determinant of cardiomyocyte death, making it a potential drug target in the treatment of cardiovascular ischaemic diseases. It seems that in HK-mitochondrial binding, the two human diseases with the largest impact on human mortality and morbidity, cancer and cardiovascular disease, share a similar, but directionally opposing, cellular mechanism: mtHK binding is *increased* in malignant cancer providing it with resilience against cell death, whereas it is *decreased* during cardiac infarction and thereby contributes to cardiac cell death. The challenge is now to target this mechanism that governs whether cells live or die in the treatment of cardiovascular diseases, with a minimum of collateral damage.

Conflict of interests

None.

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