UNIVERSITY OF CALIFORNIA, SAN DIEGO

Role of Mitochondrial Hexokinase-II Dissociation in the Induction of Mitophagy in Cardiomyocytes

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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2014

The thesis of Eric Yuxiao Ding is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California, San Diego

2014

DEDICATION

This thesis is dedicated to my family for their unconditional support and to all my friends, without whom I would have graduated much earlier.

Thank you.

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LIST OF ABBREVIATIONS

Akt	Protein Kinase B
BFA	Bafilomycin A
COX-IV	Cytochrome c oxidase IV
CsA	Cyclosporine A
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HK-II	Hexokinase-II
Mfn2	Mitofusin2
mPTP	Mitochondrial permeability transition pore
NRVM	Neonatal rat ventricular myocyte
PINK1	PTEN induced kinase 1
TMRE	tetramethylrhodamine, ethyl ester
Ub	Ubiquitin
VDAC	Voltage-dependent anion channel

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Figures 3 and 4 are reprints of materials as they appear in Roberts, D.J., V.P. Tan-Sah, J.M. Smith, and S. Miyamoto, Akt phosphorylates HK-II at Thr-473 and increases mitochondrial HK-II association to protect cardiomyocytes. J Biol Chem, 2013. 288(33): p. 23798-806. The thesis author has minor experimental contributions to this paper.

ABSTRACT OF THE THESIS

Role of Mitochondrial Hexokinase-II Dissociation in the Induction of Mitophagy in Cardiomyocytes

by

Eric Yuxiao Ding

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Hexokinase-II (HK-II), the first glycolytic kinase, is found not only in the cytosol, but also at mitochondria. We previously showed that interventions increasing mitochondria associated HK-II are cardioprotective. In this study, we tested the

hypothesis that mitochondrial HK-II regulates mitochondria specific autophagy (mitophagy), removing damaged mitochondria by lysosomal degradation to preserve cellular integrity. We showed that mitochondrial HK-II is significantly decreased in the whole heart as well as neonatal rat ventricular myocytes (NRVMs) when subjected to ischemia. To explore the consequences of mitochondrial HK-II dissociation, a dissociation peptide (15G) was expressed in NRVMs. Expression of 15G decreased mitochondrial HK-II by 40%. Peptide treatment also increased mitochondrial Parkin, an important mitophagic ubiquitin ligase, as well as ubiquitination of mitochondrial proteins and decreased expression of the mitochondrial protein VDAC. Expression of 15G did not decrease cytosolic or nuclear proteins, nor did it induce mitochondrial membrane depolarization. The VDAC decrease is recovered by inhibition of lysosome activity, suggesting that HK-II dissociation regulates lysosome dependent mitophagy. PINK1, a mitochondrial kinase implicated in Parkin recruitment, was not observed, suggesting that mitochondrial HK-II dissociation recruits Parkin through a mechanism distinct from the established role of PINK1 in Parkin recruitment to depolarized mitochondria. Mitofusin2 (mfn2) has been shown to be a mitochondrial Parkin receptor. Our study revealed that HK-II binds to mfn2 and that this binding is diminished by 15G expression or ischemia. Together these results suggest that HK-II dissociation plays a regulatory role in Parkin translocation and induction of mitophagy in response to ischemia in cardiomyocytes.

INTRODUCTION

Heart disease is the leading cause of death in the United States, claiming nearly 600,000 lives in 2011 alone, and ischemic heart diseases account for over 60% of these deaths[1]. Preserving cardiomyocyte integrity and heart function throughout the course of a myocardial infarction is critical to a favorable prognosis[2], and significant progress in research in the past few decades has led to better understanding and treatment options for patients of ischemic heart disease. Due to the high density of mitochondria in myocardial tissue[3] as well as their critical involvement in cell death signaling[4], mitochondrial dysfunction is a focus of studies on cardiac pathology[4-6].

Mitochondria have been long recognized as the primary source of cellular ATP generation. After glucose is broken down through glycolysis in the cytosol, the resultant pyruvates are shuttled into the mitochondrial matrix and decarboxylated to produce acetyl-CoA. This acetyl-CoA then combines with oxaloacetate and proceeds through the citric acid cycle, reducing the electron carriers NAD^+ and FAD into NADH and $FADH_2$[7]. These electrons are then used to drive oxidative phosphorylation at the inner mitochondrial membrane, pumping protons across the membrane into the intermembrane space and forming an electrochemical gradient, generating membrane potential. The protons then travel back across the inner membrane through the enzyme ATP

synthase, driving the production of ATP[8]. This entire process supplies the cell with a large amount of ATP, far exceeding that produced through glycolysis[9].

In addition to their role in metabolism, mitochondria are well recognized as initiators in many cell death pathways[4]. Mitochondrial damage has been thoroughly linked to many pathological conditions including neurodegenerative diseases[10-12], ischemic stroke, and ischemic heart diseases[2, 5, 13]. Mitochondria play a central role in the development of apoptosis and programmed necrosis in the heart. Members of the Bcl-2 family proteins tightly regulate the release of mitochondrial contents through regulation of permeability of the outer mitochondrial membrane. Pro-apoptotic Bcl-2 proteins such as Bax and Bak induce permeabilization of the outer membrane, while anti-apoptotic members including Bcl-2 and Bcl-xL antagonize this[14]. Upon permeabilization, apoptotic factors, including cytochrome c, exit the intermembrane space into the cytosol and interact with apoptosis protease-activating factor 1 (Apaf-1) to activate caspase-9, initiating a signal cascade leading to apoptosis[15]. Mitochondria also play a major role in necrotic cell death. Opening of the mitochondrial permeability transition pore (mPTP), a mega channel formed at the inner mitochondrial membrane, abolishes ATP synthesis and induces swelling of the matrix, causing rupture of the inner and outer mitochondrial membranes, and necrotic death[16]. Two established inducers of mPTP opening are reactive oxygen species (ROS) and mitochondrial calcium overload[17]. Increased cellular ROS is a causal factor in many pathophysiological settings[18].

Mitochondria are also known to be major contributors to cellular production of ROS through complexes I and III of the electron transport chain[19]. In addition, mitochondria contribute to calcium overload mediated cell death. Many cellular stresses are associated with calcium release at the endoplasmic/sarcoplasmic reticulum, which in turn leads to mitochondrial calcium uptake, and depending on the range of insult, the rise in mitochondrial calcium concentration will determine the cell's course of action[20]. A modest and transient calcium influx only triggers increased ATP production, while a sustained or large increase in calcium is a key inducer of mPTP, leading to swelling and rupture of the mitochondria, and ultimately necrosis.

Hexokinase-II (HK-II) is an isoform of the essential glycolytic enzyme expressed in insulin sensitive tissues including the heart, responsible for the phosphorylation of glucose into glucose 6-phosphate (G6P)[21]. This conversion initiates the process of glycolysis and commits the cell to using glucose for ATP generation. HK-II contains a mitochondrial localization sequence on its N-terminus[22], and mitochondrial association is theorized to confer preferential access to ATP, maximizing enzyme efficiency. Due to the heightened importance of glycolysis in cancer, HK-II has been well studied in many cancer cell lines. It has been demonstrated that HK-II is upregulated in many types of tumors and studies have revealed that mitochondria associated HK-II has protective effects against apoptotic and necrotic insults in cancer cells[21, 23]. In more recent years, work on HK-II has also been extended to its protective role

in the heart. We and others have shown that mitochondrial HK-II binding stabilizes the outer mitochondrial membrane against permeabilization and prevent mPTP formation in cardiomyocytes[17]. We also demonstrated that Akt, a strong protective kinase, phosphorylates HK-II and increase its mitochondrial binding to preserve mitochondrial integrity against oxidative stress[24]. Increased mitochondrial association of HK-II has also been shown to contribute to cardioprotective effects of ischemic preconditioning (IPC) against ischemia-reperfusion (IR) injury[13, 22, 25-28] as well as to alleviate cardiac hypertrophy and remodeling[25], precursors to heart failure. Most studies aimed at demonstrating the role of mitochondrial HK-II have utilized the mitochondrial binding domain of HK-II (N-terminus) fused with cell permeable peptide sequences such as TAT[29, 30], (TAT-HK-II) to competitively bind at mitochondria and dissociate bound HK-II.

Autophagy was initially understood as a programmed death pathway in which cellular components are digested through lysosomal degradation[31]. However, recent research revealed that autophagy can contribute to preservation of cellular homeostasis by catabolizing cellular components for energy in response to nutrient starvation[31-33]. It also serves as a pathway for the elimination of dysfunctional units within the cell and thereby limits propagation of cellular damage. The Atg family proteins, which are very well conserved and regulated across many organisms, are critical in this process. They mediate the formation and expansion of a double membrane structure

known as a phagophore, which engulfs the target of degradation, forming an autophagosome. The autophagosome then fuses with a lysosome, leading to degradation of its contents[32, 33]. Autophagy of specific organelles has in recent years become an increasingly popular field of research. Autophagy of mitochondria (mitophagy) was traditionally viewed as an energetic process. Mitophagy has now been established to be a critical process in quality control and maintenance of overall mitochondrial integrity, to minimizing perpetuation of deleterious mitochondrial death signaling in response to stress[34, 35].

PTEN-induced kinase 1 (PINK1) is an enzyme that localizes to mitochondria and is transported to the inner membrane, where it is cleaved by mitochondrial proteases and subsequently degraded. In a depolarized mitochondrion, however, neither the transport nor processing steps occur and full-length PINK1 begins to accumulate on the outer membrane[36, 37]. The presence of intact PINK1 acts as a sensor for depolarized mitochondria and serves to recruit the E3 ubiquitin ligase Parkin to mitochondria. Parkin then ubiquitinates various mitochondrial proteins, essentially flagging the damaged mitochondrion for removal[36, 38]. The ubiquitin chains are recognized by the autophagic adapter protein p62 which binds to LC3 and facilitates autophagosome recruitment, engulfing the damaged mitochondrion. Finally, like in general autophagy, fusion of the autophagosome with a lysosome eliminates the contents through enzymatic degradation. Mitophagy as a process eliminates damaged mitochondria before the effects of stress signals

can induce programmed cell death, and has accordingly been reported to be involved in cardioprotection[6, 39, 40].

Although the importance of PINK1 and Parkin in induction of mitophagy has been well documented, the substrates for PINK1 and binding sites for Parkin at mitochondria were not fully determined. A recent seminal study reported that the outer membrane fusion protein mitofusin2 (mfn2) is phosphorylated by PINK1 and serves as a mitochondrial binding partner for Parkin[38]. Mfn2 is a key protein in mitochondrial turnover, and serves an important role in the tethering of two mitochondria prior to fusion as well as actual fusion of the outer membranes. In the context of mitophagy, knockout of mfn2 has been reported to cause deficiencies in Parkin mediated mitochondrial ubiquitination. Due to the fact that IR both dissociates mitochondrial HK-II[28] as well as induces mitochondrial Parkin translocation and mitophagy[40], we hypothesized that HK-II dissociation may have a regulatory role in induction of mitophagy. We propose that HK-II also binds to mfn2, and that disrupting this association vacates mfn2 for Parkin to bind, thereby facilitating mitophagy.

Cell Culture

Culture dishes were coated with 1% gelatin overnight and washed prior to myocytes plating. Neonatal rat ventricular myocytes (NRVM) were isolated with the neonatal cardiomyocyte isolation system (Worthington) and plated at 1 x 10^6 cells / 6 cm and 3 x 10^6 cells / 10 cm. Cardiomyocytes were maintained in 4:1 Dulbeco's modified Eagle's medium (DMEM)/medium199 (Invitrogen) with 15% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) after plating. Cells were serum starved prior to any treatment with DMEM high glucose supplemented with antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin).

Isolated perfused (Ex-vivo) ischemia

Hearts were excised from age-matched male mice and perfused in a retrograde manner with modified Krebs-Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM EDTA, 1.2 mM $MgSO_4$, 11 mM glucose, 1.5 mM Na-Pyruvate, and 2 mM $CaCl_2$) in a Langendorff apparatus at 37 °C at constant pressure (80 mmHg). Hearts were stabilized for 30 minutes and then subjected to 30 minutes of global ischemia by stopping perfusion.

Simulated (In-vitro) ischemia

Cells were incubated in an ischemia-mimetic buffer (125 mM NaCl, 8 mM KCl, 20 mM 2-deoxyglucose, 0.5 mM Na-Lactate, 1.25 mM $MgSO_4$, 1.25

mM $CaCl_2$, 1.2 mM KH_2PO_4, 6.25 mM $NaHCO_3$, 20 mM HEPES, pH 7) and placed in an anaerobic chamber. The chamber was filled with a gas mixture of 95% N_2 5% CO_2, sealed, and incubated at 37^oC for 20 or 60 minutes.

Cytosol/Mitochondria fractionation

NRVMs were used to prepare cytosol and mitochondrial fractions. Cardiomyocytes were washed twice with phosphate buffered saline (PBS) and harvested with PBS into a conical tube on ice. Heart tissue was homogenized using a Tissuemiser (Fisher Scientific). Cells or tissue samples were centrifuged at 900 rcf at 4^oC for 2 minutes and supernatant was discarded. The pellet was resuspended with 50 µl buffer A (20mM HEPES, pH 7.4) and shaken on an orbital shaker at 600 rpm for 2 minutes, and 10 µl 1/16 buffer B (8% CHAPS) was added before resuming shaking at 600 rpm for 5 minutes. 60 µl of buffer C (420mM Mannitol, 140mM sucrose, 2mM EGTA in Buffer A, pH to 7.4) was then added and then samples were centrifuged at 700 rcf for 10 minutes at 4^oC. Supernatant was transferred to clean microfuge tubes and the pellet discarded. These tubes were then centrifuged at 12,000 rcf for 15 minutes at 4^oC, and the supernatant once again removed and saved as the cytosolic fraction. The remaining pellet is washed once with 200 µl of a 1:1 solution of buffer A and buffer C and resuspended in 60 µl RIPA lysis buffer then centrifuged again at 16,000 rcf. The supernantant was removed and added to the cytosolic fraction while the pellet is resuspended in 60 µl RIPA buffer supplemented with 200 µM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PNPP and 1 mM PMSF and labeled as the mitochondrial

fraction. Both fractions are sonicated on ice for 1 minute and protein concentration was measured according to the Micro BCA Protein Assay Kit protocol (Thermo Scientific). Samples were prepared using Invitrogen NuPAGE LDS Sample Buffer and reducing agent, then heated for 15 minutes at 100^{o}C and centrifuged for 30 seconds prior to loading.

Adenoviral infection

NRVMs were infected with adenovirus to overexpress green fluorescent protein (GFP), wildtype hexokinase II (WT HK-II), and 15 amino acids of the N terminus of hexokinase II fused with GFP (15G). The 15G adenovirus was infected at 50, 150, and 300 MOI, referred to as low, medium, and high doses. Cells were infected for 24 hours, and washed 3 times with DMEM high glucose supplemented with antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin).

Immunoprecipitation

Cardiomyocytes were washed 3 times with ice-cold PBS and then harvested in 500 µl of 1% CHAPS buffer (1% CHAPS, 5 mM EDTA, pH 7.4) supplemented with 200 µM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM p-nitrophenyl phosphate (PNPP) and 1 mM phenylmethysulfonyl fluoride (PMSF). Lysates were collected in a microfuge tube and nutated for 20 minutes at 4^{o}C, then centrifuged at 20,000 x *g* for 10 minutes at 4^{o}C. Protein concentration was measured using Micro BCA Protein Assay Kit (Thermo Scientific), protein was normalized, and the supernatant was then transferred to a clean microfuge tube and primary antibody targeting the protein of interest

was added. Protein A/G Agarose beads (Santa Cruz) were washed 3 times with 1 ml 1% CHAPS buffer, mixed well, and 30 µl of 50% beads slurry were added to each tube. Tubes were nutated overnight at 4 ^{o}C, then supernatant was removed and the beads were washed 3 times with 1 ml 1% CHAPS buffer. 60 µl 3x Invitrogen NuPAGE LDS Sample Buffer was added and samples were boiled at 100^{o}C for 15 minutes. Tubes were vortexed and centrifuged, and 55 µl of supernatant was transferred from each tube into a new set of sample tubes.

Western Blot Sample Preparation

Cardiomyocytes were washed 3 times with PBS and then harvested in 80 µl of RIPA buffer composed of 150 mM NaCl, 50 mM Tris (pH7.4), 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.2 mM ethylenediaminetetraacetic acid (EDTA) supplemented with 200 µM Na_3VO_4, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PNPP and 1 mM PMSF. The lysates were collected into microfuge tubes and sonicated on ice for 1 minute, nutated for 10 minutes at 4^{o}C, then centrifuged at 20,000 x *g* at 4^{o}C for 10 minutes. The supernatant was then transferred into a clean microfuge tube. Protein concentration was measured using Micro BCA Protein Assay Kit (Thermo Scientific), protein was normalized, and samples were prepared using Invitrogen NuPAGE LDS Sample Buffer and reducing agent, heated for 15 minutes at 80^{o}C and centrifuged for 30 seconds.

Western Blotting

Invitrogen NuPAGE Bis-Tris gels were loaded with an equivalent amount of protein (7-10 µg) and run at 120 volts for 2 hours. PVDF membranes (Millipore) were placed in methanol for 1 minute, then transfer buffer. Protein was transferred onto the PVDF membranes at 120 volts for 2 hours. Blot were blocked with 5% milk in tris-buffered saline-tween (TBS-T) for 1 hour and then probed overnight with 1:1000 primary antibody dilutions in 5% Bovine Serum Albumin (BSA)/TBS-T containing 0.02% NaN_3. Blots were washed with TBS-T 3 times for 5 minutes each and the incubated with secondary antibody (1:2000-1:4000 dilution) in 5% milk/TBS-T for 1 hour. Blots were then visualized with SuperSignal West Femto (Thermo Scientific) as substrate for the horseradish peroxidase conjugated secondary antibody. FluorChem Imaging software was used to develop the Western Blots and band intensity was quantified using AlphaView software.

Statistical Analysis

Band intensities were corrected for loading by normalizing to loading control (α-actinin, GAPDH). Data obtained from the same treatment conditioned were averaged and fold over control was calculated for each. The standard error of the mean (S.E.M.) was calculated, and significance data was obtained through the student's t-test using two-tailed distribution or ANOVA. Probability < 0.05 was considered to be significant.

RESULTS

Simulated ischemia dissociates mitochondrial HK-II.

Mouse hearts were perfused in the Langendorff mode and subjected to 30 minutes of no-flow ischemia. Mitochondrial fractions were isolated to determine the level of mitochondrial HK-II. As previously reported, mitochondrial binding of HK-II was significantly decreased by ischemia (Figure 1A). We also examined the effect of simulated ischemia on HK-II distribution in neonatal rat ventricular myocytes (NRVMs). As observed in the ex-vivo model, mitochondrial HK-II was significantly decreased by ischemic stress (Figure 1B).

Characterization of the 15G peptide.

The N-terminus of HK-II is well established as its mitochondrial binding domain. We expressed wild-type (WT) HK-II or an N-terminus deletion mutant of HK-II (ΔN) in NRVMs and demonstrated that both these HK-II constructs lead to increased cytosolic HK-II expression. The WT HK-II was also expressed at mitochondria, but ΔN was not (figure 2).

In order to further determine the consequences of mitochondrial HK-II dissociation, as with ischemia, we generated a peptide (15G) consisting of the N-terminus of HK-II fused with green fluorescent protein (GFP), which should bind to mitochondria and competitively inhibit HK-II binding to mitochondria. For initial characterization of the 15G peptide, NRVMs were infected with 15G

adenovirus for 24 hours and GFP fluorescence was visualized. We used TMRE (tetramethylrhodamine, ethyl ester), a mitochondrial membrane potential indicator, to visualize functional mitochondria. When compared to the GFP expressing adenoviral control (Ad-GFP), the GFP tagged 15G peptide showed co-localization with TMRE fluorescence (Figure 3), indicating that the 15G peptide localizes to mitochondria. To determine whether 15G peptide could dissociate HK-II from mitochondria, mitochondrial fractions were isolated from NRVMs infected with low (50 MOI; L), medium (150 MOI; M), or high (300 MOI; H) doses of the 15G adenovirus for 24 hours. Western blot analysis showed a strong dose dependent decrease in mitochondrial HK-II levels upon expression of the 15G peptide (Figure 4), demonstrating that expression of 15G dissociates HK-II from mitochondria.

Forced dissociation of HK-II from mitochondria causes loss of mitochondrial proteins.

The 15G peptide was expressed in NRVMs via adenoviral infection for 48 hours at low and medium doses and whole cell lysate prepared to see the effects of mitochondrial HK-II dissociation. Unexpectedly, 15G treatment decreased levels of the outer mitochondrial membrane protein VDAC and the inner membrane protein COX-IV (Figure 5A). The cytosolic protein GAPDH and nuclear protein Lamin A/C were not changed (Figure 5B) The decrease in both outer and inner mitochondrial membrane proteins suggests mitochondrial degradation is induced by expression of 15G.

Mechanism for 15G induced loss of mitochondrial protein.

Lysosomal degradation is an established final step in autophagy and mitophagy. To determine whether lysosomal degradation contributes to the decrease in mitochondrial protein induced by expression of 15G, we examined the effect of blocking lysosomal activity. Lysosomal inhibition achieved by the addition of bafilomycin A (30 μM) prevented the decrease in VDAC levels induced by 15G expression (Figure 6) and attenuated the decrease in COX-IV (data not shown). These results suggest that lysosomal degradation of mitochondria contributes to the decrease in mitochondrial proteins induced by mitochondrial HK-II dissociation.

Moderate dissociation of mitochondrial HK-II potentiates FCCP induced ubiquitination of mitochondria.

Ubiquitination of mitochondrial proteins serves as a signal for the recruitment of autophagosomes and is therefore a key step in the process of mitophagy. To determine whether 15G treatment leads to ubiquitination of mitochondrial proteins, cells were infected with GFP or 15G at the low dose for 24 hours, and mitochondrial fractions were isolated and examined by Western blotting for ubiquitin. Ubiquitin Western blot analysis in mitochondrial fractions showed a slight increase in mitochondrial ubiquitination with 15G. Low levels of 15G only moderately dissociates HK-II (~20%), thus we co-expressed 15G with FCCP (carbonilcyanide p-trifluoromethoxyphenylhydrazone), a mitochondrial uncoupler which depolarizes mitochondria and is commonly

used to induce mitophagy. Treatment of NRVMs with FCCP at 10 μM for 6 hours increased mitochondrial ubiquitination in control (GFP) cells, as previously reported in both non-cardiac and cardiac cells (Figure 7). Remarkably, this response was greatly enhanced by expression of 15G. Overexpression of HK-II, which had minimal effect when used alone, had the converse effect, decreasing the ubiquitination induced by FCCP (Figure 7). These results implicate that a modest amount of mitochondrial HK-II dissociation facilitates ubiquitination in damaged/depolarized mitochondria.

Increased mitochondrial HK-II dissociation is sufficient for mitochondrial protein ubiquitination and induces Parkin translocation to the mitochondria.

We then determined the effects of increasing HK-II dissociation by expression of 15G at a medium dose which dissociates 40% HK-II from mitochondria. 15G expression at 150 MOI for 24 hours was sufficient to induce massive ubiquitination of mitochondria (Figure 8A) even in the absence of uncoupling. Parkin is a ubiquitin ligase responsible for mitochondrial ubiquitination. As shown, Parkin was significantly increased in the mitochondrial fraction isolated from cells expressing 15G (Figure 8B). This response was largely inhibited by co-expression of wildtype HK-II with 15G (Figure 8C), supporting the conclusion that HK-II dissociation from mitochondria is responsible for 15G induced Parkin translocation to mitochondria.

Inhibition of mPTP formation has no effect on mitochondrial Parkin translocation, ubiquitination, or protein decrease.

Mitochondrial depolarization, often induced by mPTP, is an important step in the established pathway of mitophagy. To test the role of mPTP and depolarization in ubiquitination and Parkin translocation to mitochondria induced by 15G, NRVMs were infected with GFP or 15G adenovirus for 24 hours and treated overnight with 1 μM cyclosporine A to block the formation of mPTP. Mitochondria were isolated and subjected to ubiquitin Western blotting. There was no change in mitochondrial Parkin or ubiquitination in 15G expressing cells treated with CsA (Figure 9A). We also examined the effect of mPTP inhibition by CsA on 15G induced VDAC decrease. GFP or 15G expressing NRVMs were treated overnight with 10 μM CsA and subjected to Western blot analysis for VDAC. Similar to Parkin and ubiquitination, VDAC levels remained unchanged with the addition of CsA (Figure 9B). These data provide further support for the conclusion that mPTP does not play a role in 15G mediated Parkin translocation, ubiquitination, or subsequent protein degradation.

15G expression does not induce accumulation of full-length PINK1 at mitochondria.

It has been demonstrated that PINK1, a mitochondrial kinase, is constantly degraded and thus full length PINK1 is not detectable in polarized, healthy mitochondria. In response to mitochondrial depolarization, however,

full length, functional PINK1 is accumulated and plays an important role in Parkin translocation to mitochondria. To confirm PINK1 indeed accumulates at mitochondria in response to depolarization, NRVMs were subjected to Western blot analysis for full-length PINK1. Overnight FCCP treatment (10 μM) increased PINK1 in the mitochondrial fraction as previously reported (Figure 10A). To determine whether dissociation of HK-II leads to PINK1 accumulation in mitochondria, NRVMs were infected with GFP or 15G adenovirus for 24 hours followed by fractionation and Western blotting for full length PINK1. FCCP was used as a positive control for depolarization induced PINK1 accumulation. FCCP treatment increased PINK1 in the mitochondrial fraction as previously shown. In contrast, no increase in PINK1 was observed in mitochondria isolated from cells expressing 15G (150 MOI) (Figure 10B). Our data indicate that 15G induced mitochondrial HK-II dissociation increases Parkin accumulation at mitochondria and that this is independent of mitochondrial membrane depolarization or PINK1 stabilization.

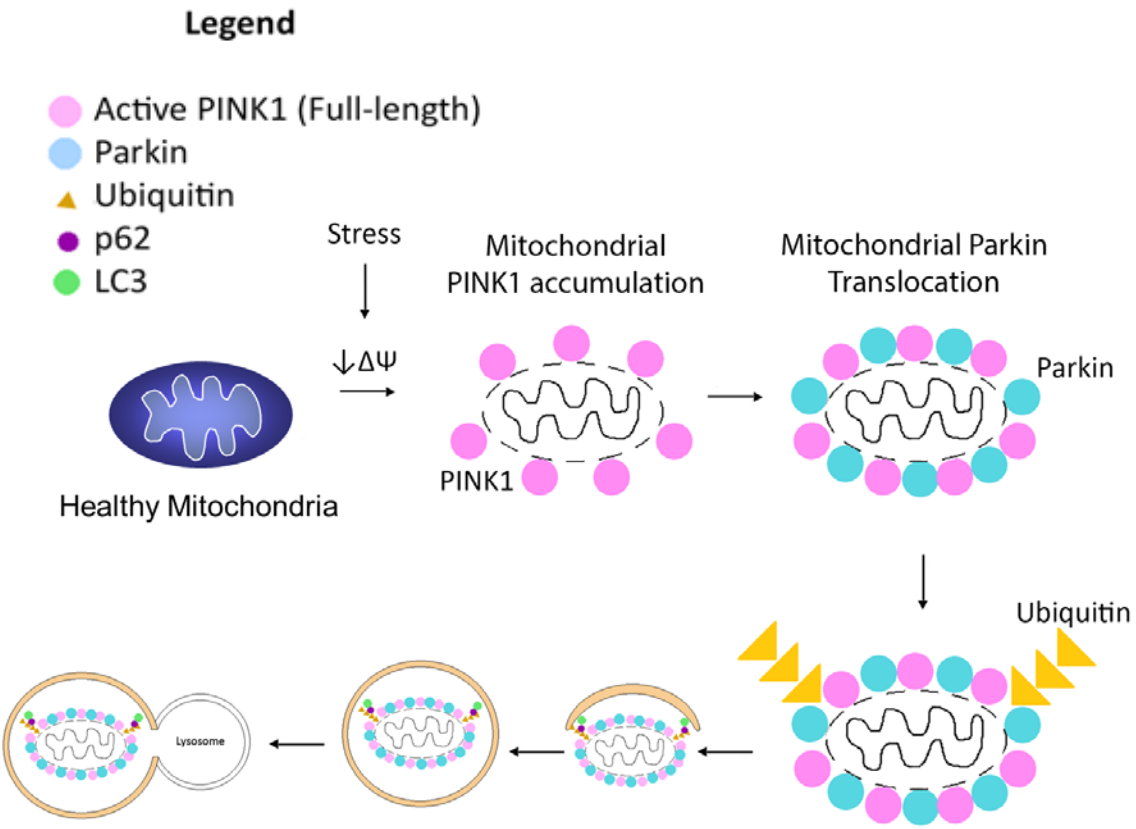
Mitofusin2 is a binding site at mitochondria for HK-II.

Mitofusin2 (mfn2) is an outer membrane protein that has been reported to be phosphorylated by PINK1 and functions as a binding site for Parkin. We hypothesized that HK-II might bind to mfn2 at mitochondria, and that the dissociation of HK-II vacates mitochondrial mfn2 for Parkin binding, thereby facilitating ubiquitination and subsequent degradation of mitochondria. First, we examined this possibility by overexpressing GFP or HK-II conjugated to

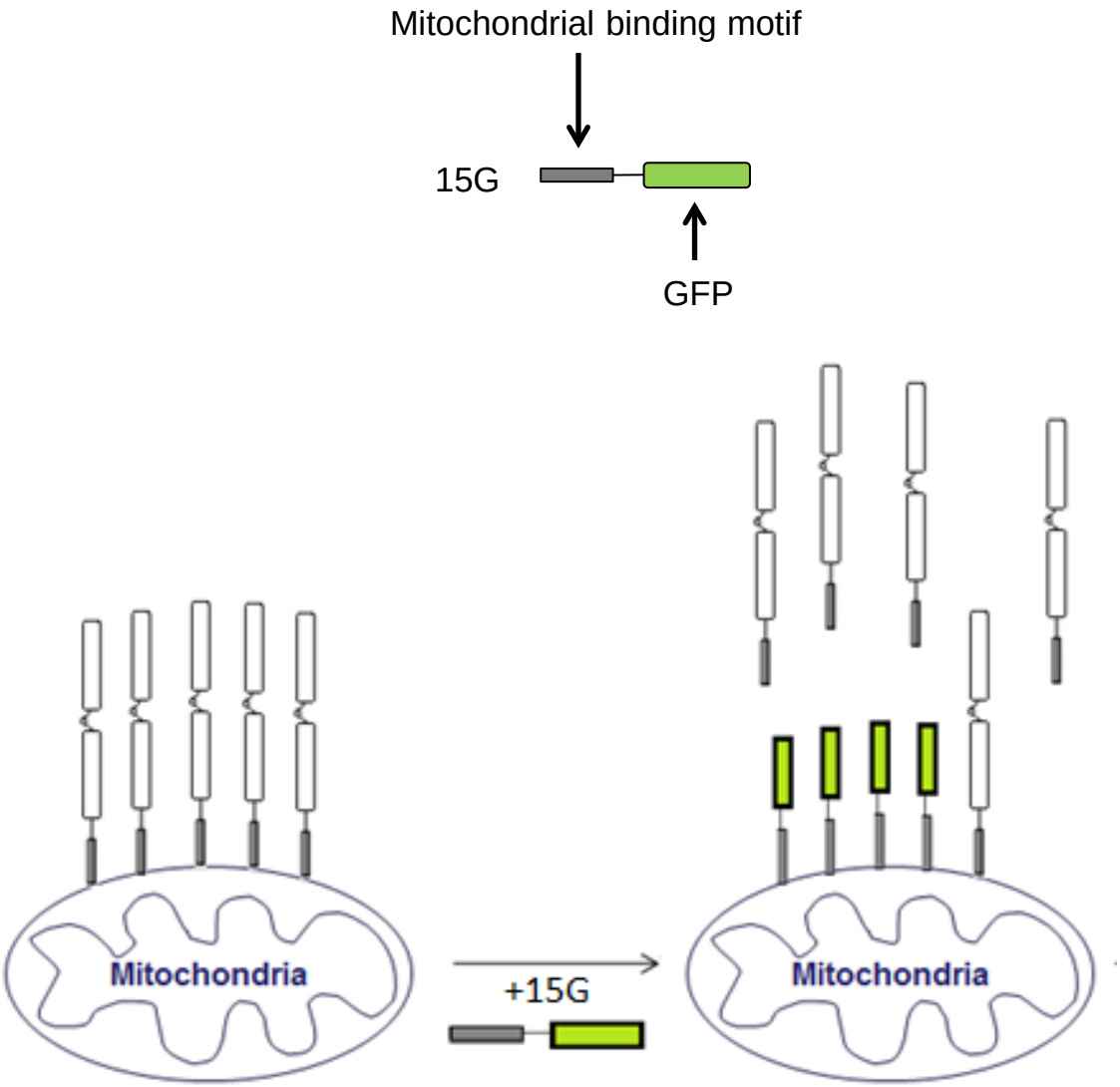
GFP (HK-II-GFP) in NRVMs and performing immunoprecipitation for mfn2 followed by Western blotting for GFP. HK-II, as assessed by GFP Western blotting, was found in the mfn2 immunocomplex (Figure 11A), suggesting an association of HK-II with mfn2. As shown earlier (Figure 1B), simulated ischemia induces mitochondrial HK-II dissociation. When NRVMs were subjected to simulated ischemia for 60 minutes, the association between overexpressed HK-II and mfn2 was decreased (Figure 11A). We then examined binding between HK-II and mfn2 with endogenous proteins. First, we examined the association of endogenous HK-II with mfn2 in control (GFP) NRVMs. HK-II was immunoprecipitated and Western blotting for mfn2 showed abundant mfn2 in the HK-II immunocomplex (Figure 11B), suggesting an interaction of these molecules at endogenous level of expression. The level of mfn2 in the HK-II immunocomplex was then determined in cells treated with 15G to block mitochondrial HK-II association. Expression of 15G markedly reduced the association of HK-II with mfn2 (Figure 11B). We then examined the effect of simulated ischemia, which also dissociates HK-II from mitochondria, on endogenous HK-II and mfn2 association (Figure 11 C and D). HK-II was immunoprecipitated from control cells or cells subjected to simulated ischemia for 20 or 60 min and blotted for mfn2 (Figure 11C). The association of HK-II with mfn2 was significantly decreased by simulated ischemia. The reverse immunoprecipitation/Western blotting experiments also revealed significant HK-II association with mfn2 and decreases after 60 min simulated ischemia (Figure 11D). These data strongly suggests that

mitochondrial HK-II serves as a binding partner for mitofusin and that this interaction can be regulated by ischemic insult.

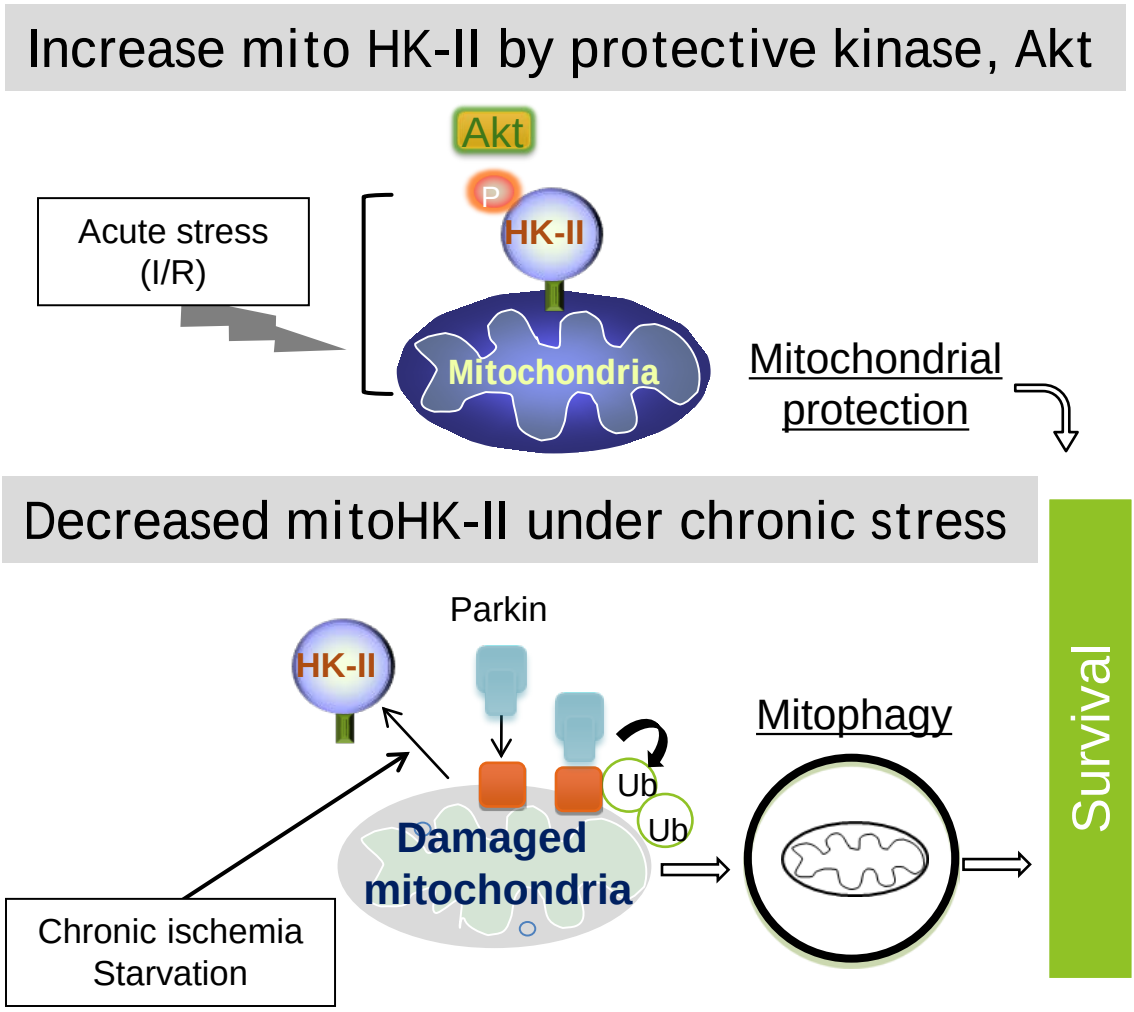
SCHEMES



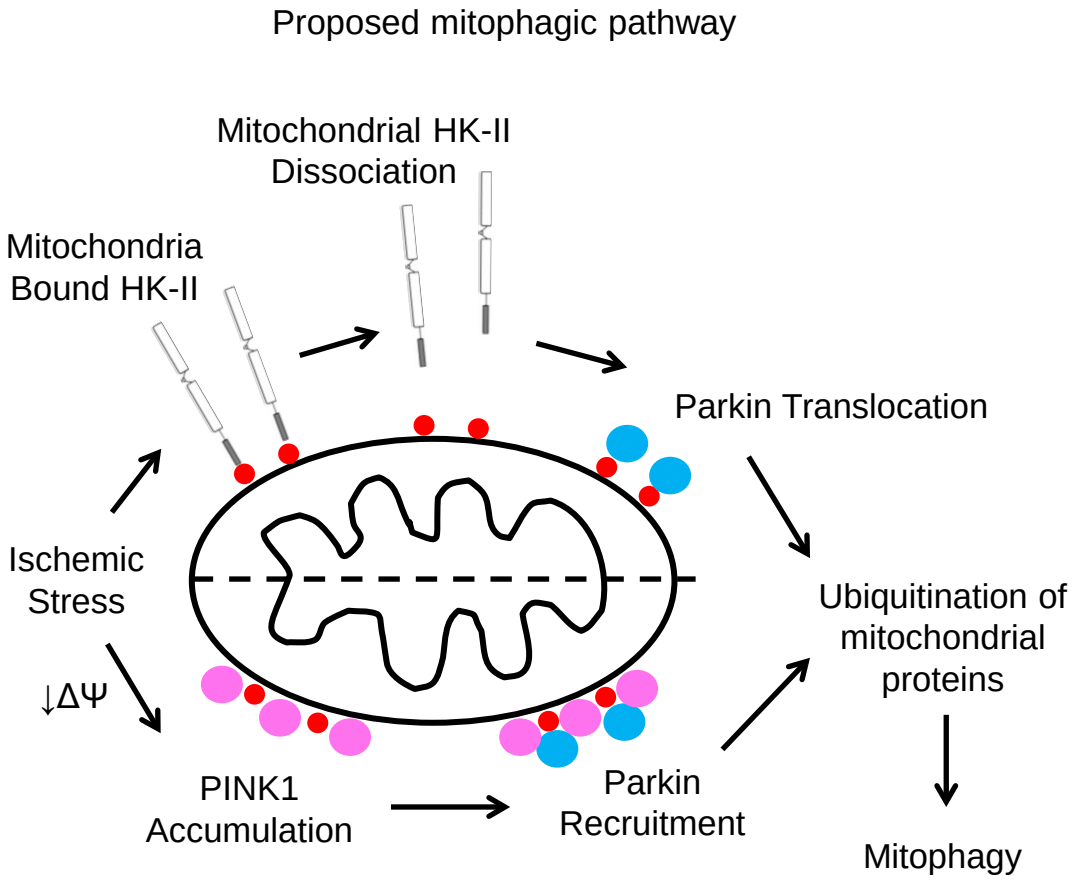
Scheme 1. Depolarization and PINK1 mediated mitophagy. Upon membrane depolarization, PINK1 cleavage ceases and consequent mitochondrial PINK1 accumulation causes Parkin translocation to the mitochondrion. Parkin then ubiquitinates various mitochondrial targets, which are recognized by the adapter protein p62, recruiting the LC3-autophagosome complex to the damaged mitochondrion. The mitochondrion is then engulfed by the autophagosome, which fuses with a lysosome to undergo degradation.



Scheme 2. The 15G HK-II dissociation peptide. The N-terminus of HK-II is responsible for mitochondrial localization. We fused this mitochondrial binding motif with GFP and subcloned it into an adenoviral vector. This peptide is used in our studies to dissociate HK-II from mitochondria. By dissociating mitochondrial HK-II through expression of the 15G peptide, we are able to observe potentially regulatory roles HK-II plays in the induction of mitochondrial autophagy.



Scheme 3. HK-II is an important regulator of mitochondrial integrity. Under acute oxidative stress, Akt phosphorylates HK-II and increases mitochondrial HK-II binding, which has been previously shown to confer mitochondrial protection and promote cell survival. Chronic ischemic damage and starvation are known to dissociate mitochondrial HK-II and induce Parkin translocation and subsequent mitophagy.



Scheme 4. Ischemia induced pathways of mitophagy. Ischemic insult is known to depolarize mitochondria, inducing PINK1 accumulation and recruiting Parkin to mitochondria. Ischemia has also been shown to dissociate mitochondrial HK-II, and we propose that this dissociation vacates HK-II from mitochondrial mfn2, vacating it to be available for Parkin binding. Both pathways lead to mitochondrial ubiquitination and mitophagy.

FIGURES

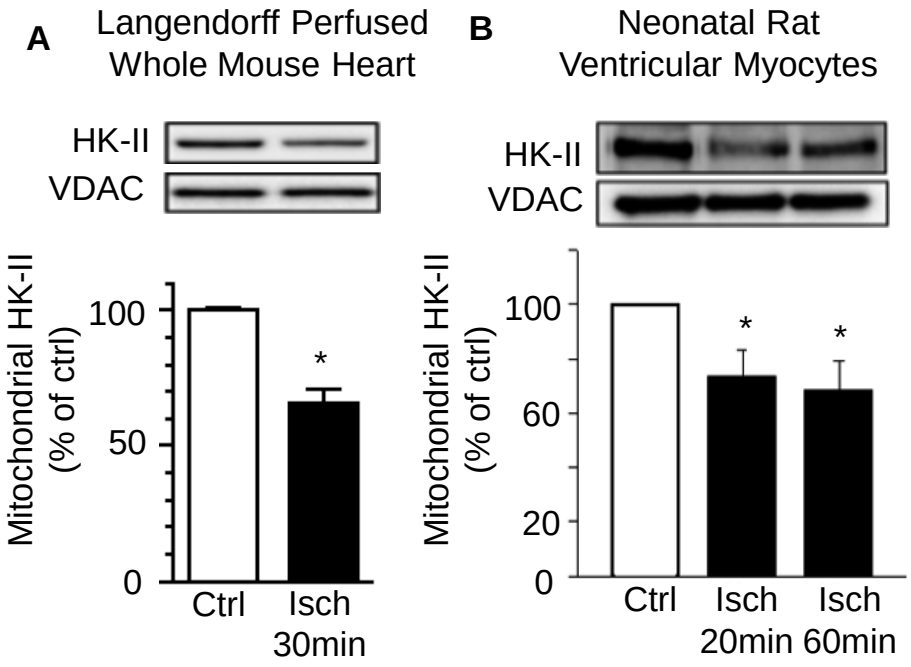


Figure 1. Ischemia induces mitochondrial hexokinase II dissociation. A. Langendorff perfused whole mouse hearts were subjected to global ischemia for 30 minutes. Tissue sample was analyzed through Western blot analysis. B. NRVMs were serum starved for 24 hours, then subjected to 20 or 60 minutes of simulated ischemia. Cells were then harvested, lysed, and Western blot analysis was performed. * $p<0.05$ vs control. n = 6 (A), 5 (B)

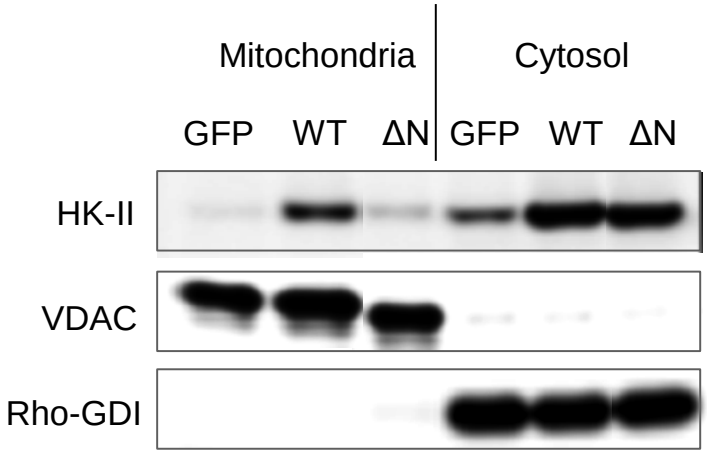


Figure 2. The N-terminus of HK-II is necessary for its mitochondrial binding. NRVM were serum starved for 24 hours prior to infection with GFP, wildtype HK-II, or N terminus deletion (ΔN*)* HK-II adenovirus. Mitochondrial-cytosolic fractionation was performed 24 hours after infection and Western blot analysis was performed.

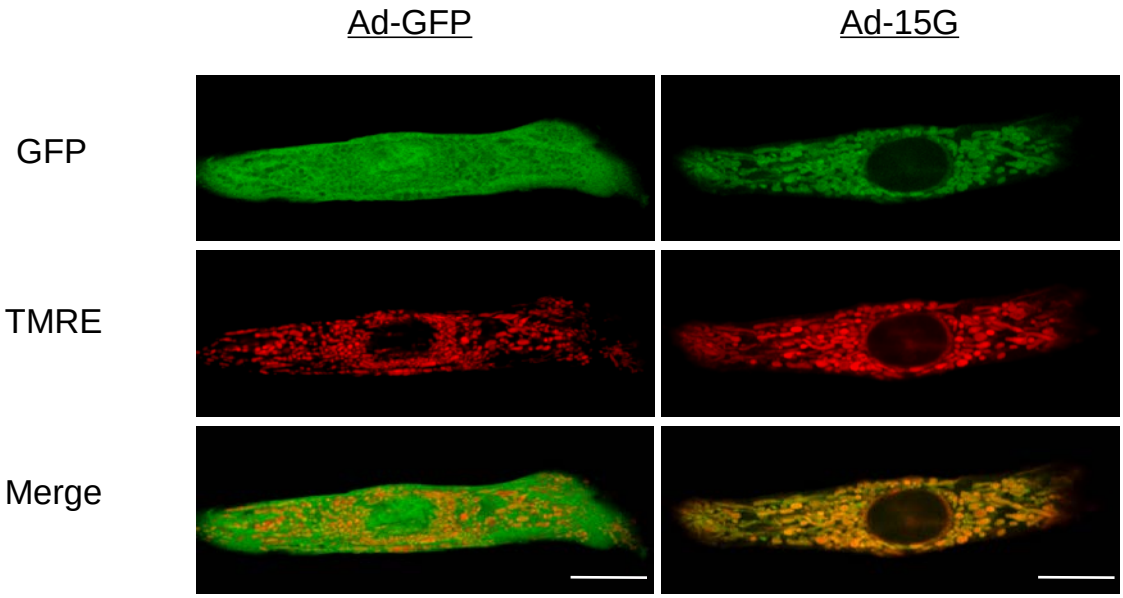


Figure 3. 15G peptide localizes to mitochondria. NRVMs were infected with either GFP or an adenoviral construct containing the 15G HK-II dissociation peptide (50 MOI). The cells were treated with TMRE to stain for active mitochondria. TMRE fluorescence was overlaid with GFP signal to visualize co-localization.

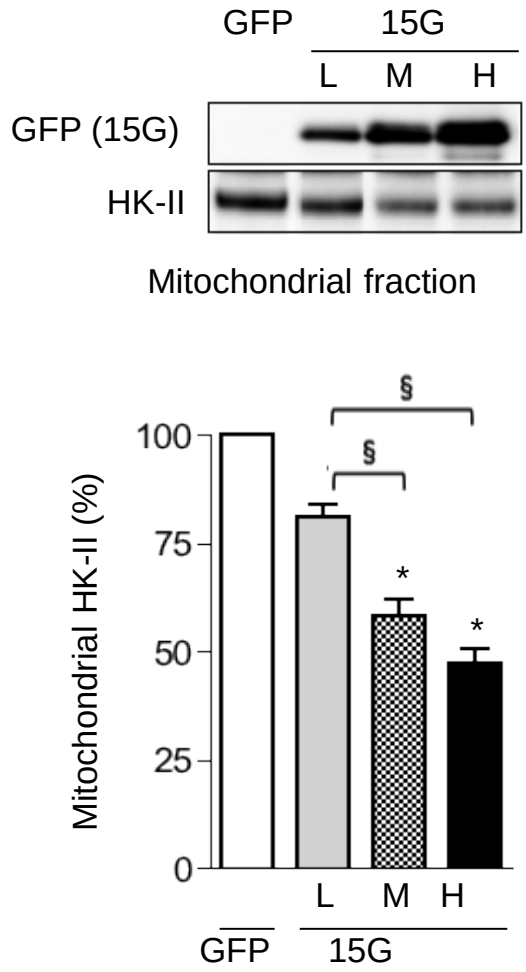


Figure 4. 15G peptide expression decreases mitochondrial binding of hexokinase-II. NRVMs were serum starved for 24 hours and then infected with GFP control or an adenoviral vector to express the 15G peptide at low, medium and high doses (50, 150, 300 MOI respectively). Mitochondrial-cytosolic fractionation was performed, and samples were subjected to Western blot analysis. *, $p<0.01$ vs GFP, §, $p<0.01$, $n = 5-6$

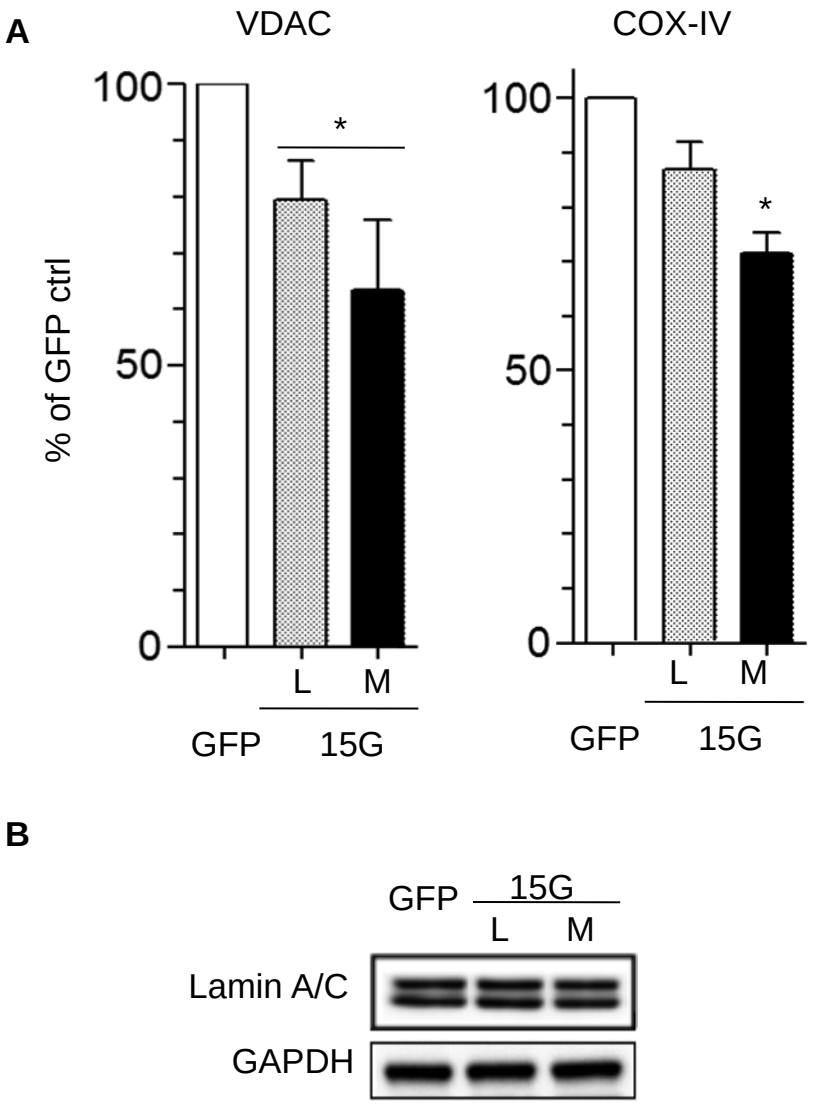


Figure 5. Mitochondrial hexokinase-II dissociation significantly lowers VDAC and COX-IV levels in whole cell lysate. NRVMs were serum starved for 24 hours and infected with 15G adenovirus at 50 (L dose) and 150 MOI (M dose). Cells were harvested and lysed 48 hours post-infection, and Western blot analysis for VDAC and COX-IV was performed. B. Lamin A/C and GAPDH remain unchanged. * $p<0.05$ vs GFP, n = 8-9

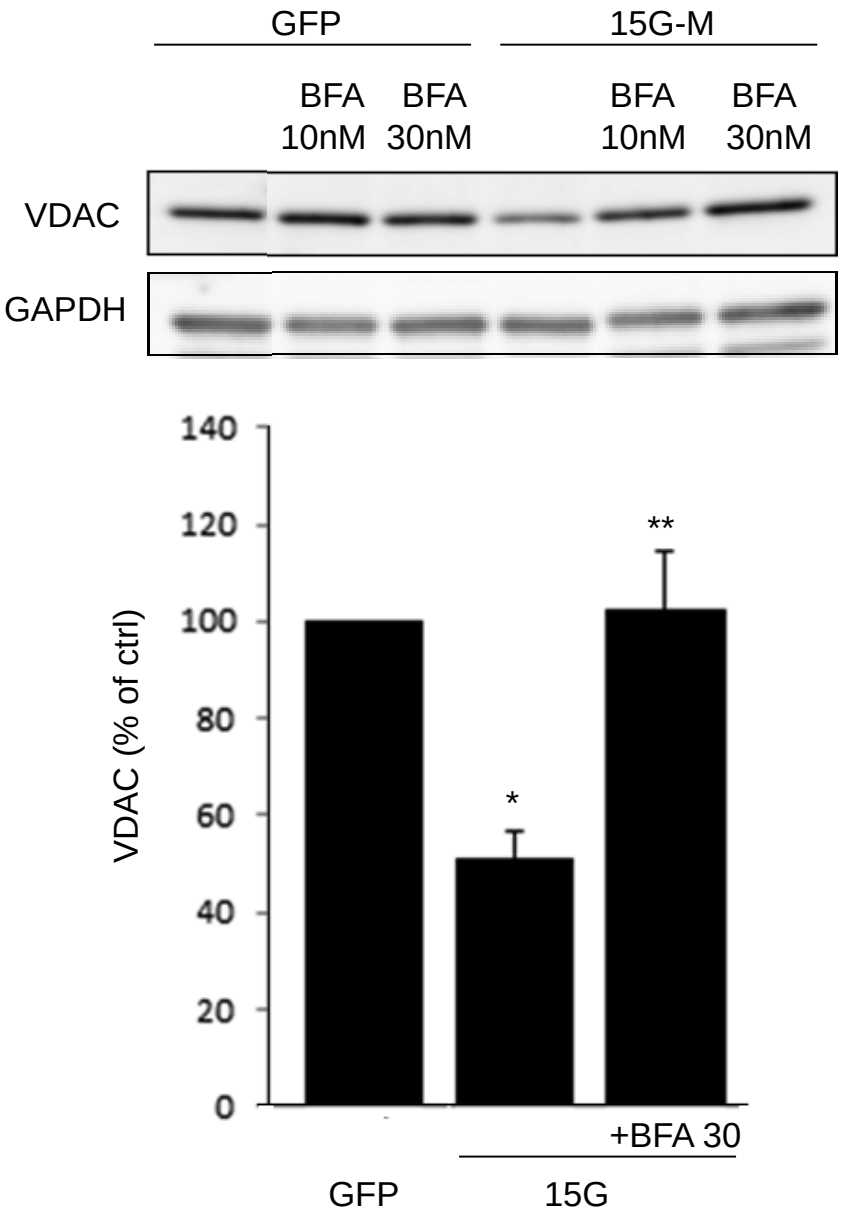


Figure 6. Bafilomycin A attenuates the decrease in VDAC induced by hexokinase II dissociation from the mitochondria. NRVMs were serum starved for 24 hours and infected with GFP control or 15G adenovirus at 150 MOI. Infected cells were treated with 10 or 30 nM bafilomycin A overnight. Cells were harvested 24 hours post-infection, lysed, and Western blot analysis was performed. * $p<0.01$ compared to GFP, ** $p<0.05$ compared to 15G, $n = 5$

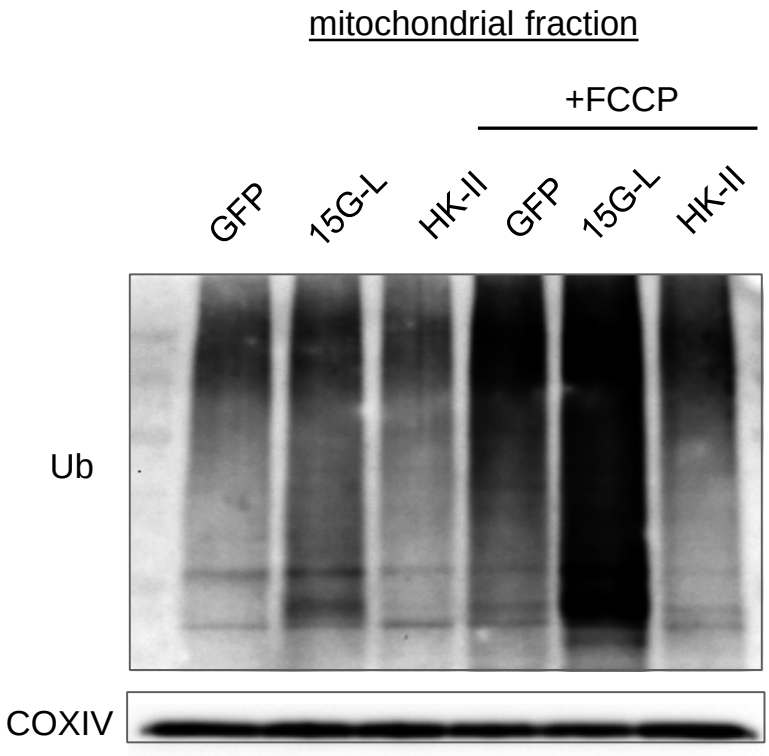


Figure 7. Moderate dissociation of mitochondrial HK-II enhances FCCP-induced ubiquitination of mitochondrial proteins. NRVMs were serum starved for 24 hours and then infected with GFP, 15G adenovirus at 50 MOI, or wildtype HK-II for 24 hours. The infected cells were then divided into either untreated or treatment with 10 μM FCCP for 6 hours. Mitochondrial fractionation was performed and samples were analyzed for ubiquitination using Western blotting.

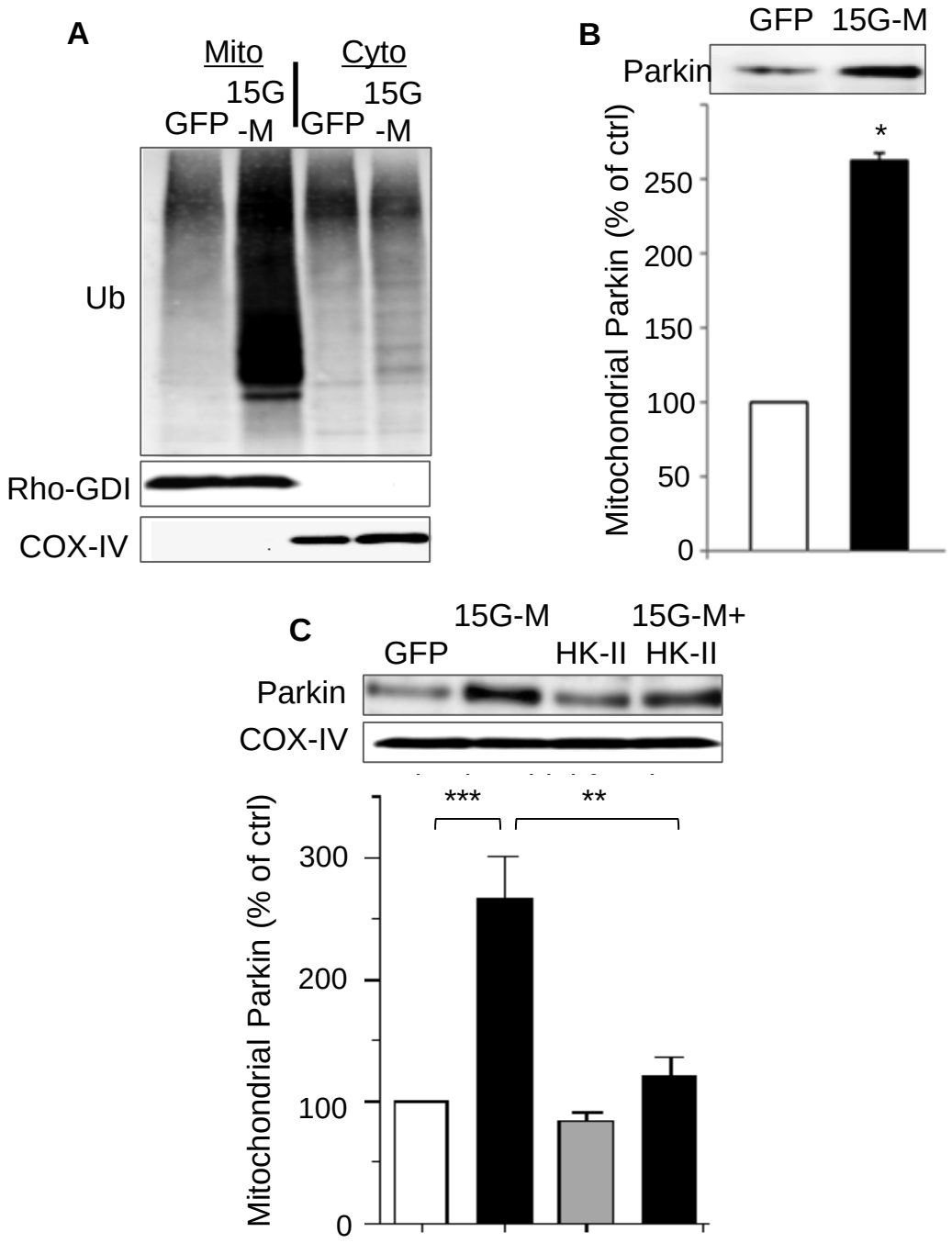


Figure 8. Increased dissociation of HK-II from mitochondria induces Parkin translocation and ubiquitination of mitochondrial proteins. NRVM were serum starved for 24 hours and then infected with GFP, 15G at 150 MOI, or HK-II adenovirus (C only) for 24 hours. Mitochondrial fractionation was performed and Parkin and ubiquitination levels were analyzed using Western blotting. *p<0.5 vs GFP, ***p<0.5 vs GFP, **p<0.5 compared to 15G, n = 3

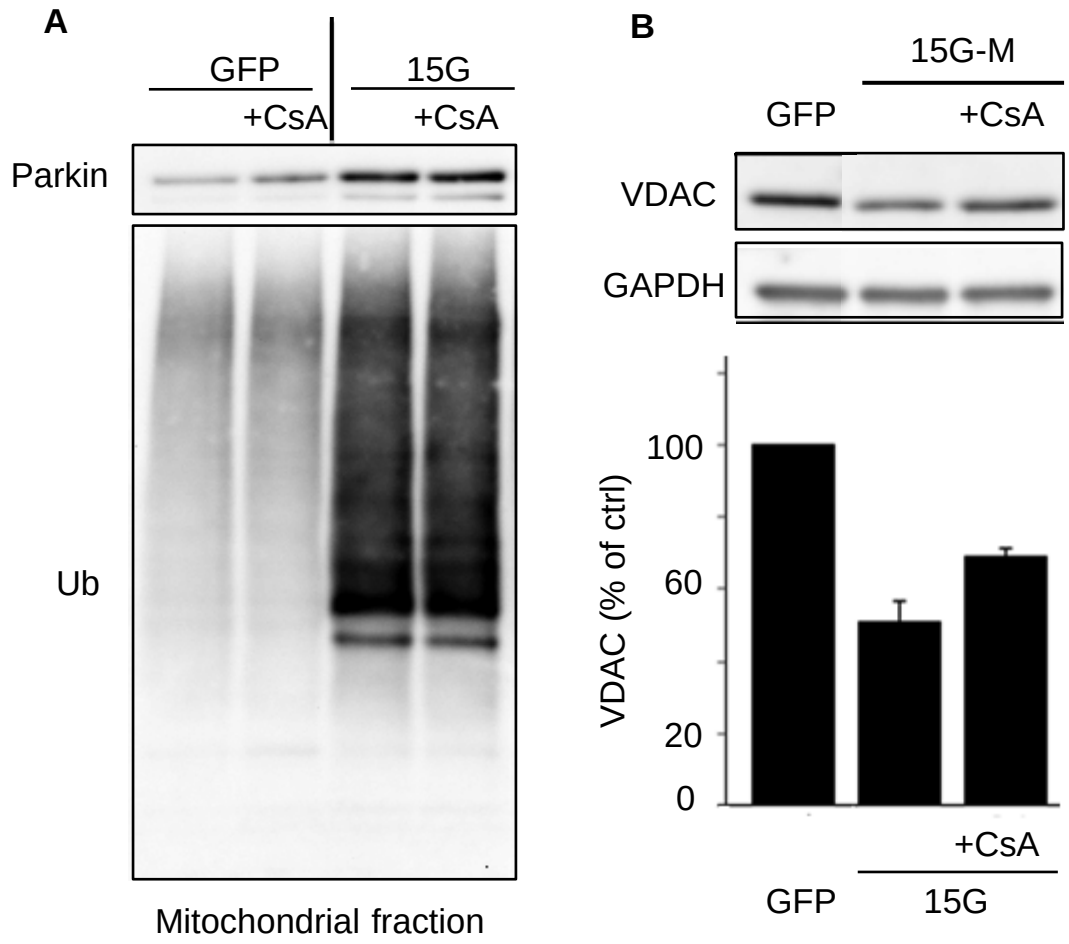


Figure 9. mPTP inhibition has no effect in mitochondrial levels of Parkin, ubiquitination, or VDAC. A. NRVM were serum starved for 24 hours and then infected with GFP or 15G at 150 MOI for 24 hours. Infected cells were divided into either untreated or treatment with 1 μM CsA overnight. Mitochondrial fractionation followed by Western blotting was performed for Parkin and ubiquitination (A) or VDAC (B) * $p<0.01$ compared to GFP, ** $p<0.05$ compared to 15G, n = 5

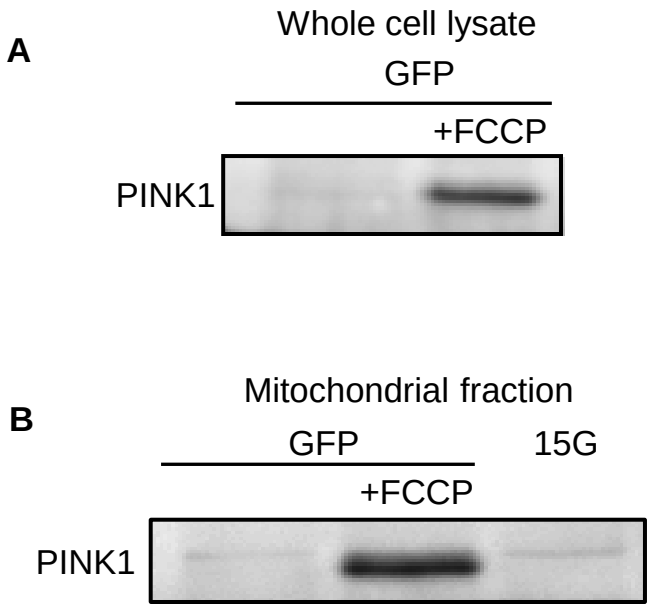


Figure 10. 15G expression does not induce accumulation of intact PINK1. NRVM were serum starved for 24 hours prior to infection with GFP or 15G for 24 hours. Infected cells were divided into either untreated or treatment with 10 μM FCCP overnight. Cells were lysed and full length PINK1 levels were analyzed using Western blotting. B. Mitochondrial fractionation was performed and full length PINK1 levels were analyzed using Western blotting.

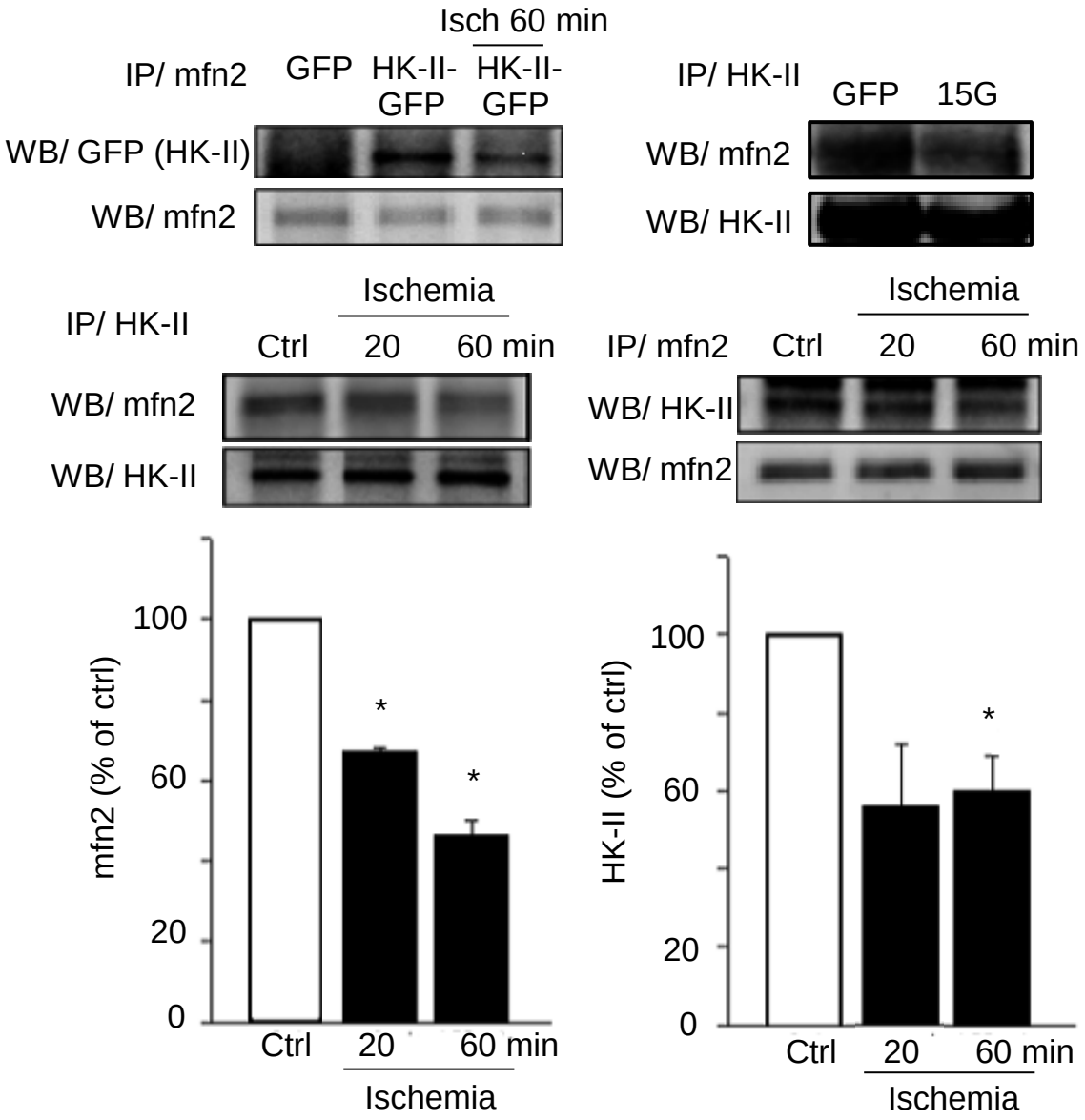


Figure 11. Mitofusin2 is a HK-II binding site at mitochondria, and this association is decreased by simulated ischemia. NRVMs were serum starved for 24 hours and then infected with GFP (A, B), HK-II-GFP (A), or 15G at 150 MOI (B). A. HK-II-GFP infected NRVMs were subjected to 60 minutes of simulated ischemia. C, D. NRVMs were subjected to 20 or 60 minutes of simulated ischemia. Cells were then harvested, lysed, and immunoprecipitation followed by Western blot analysis was performed. * $p<0.05$ vs control, $n = 3$.

Figures 3 and 4 are reprints of materials as they appear in Roberts, D.J., V.P. Tan-Sah, J.M. Smith, and S. Miyamoto, Akt phosphorylates HK-II at Thr-473 and increases mitochondrial HK-II association to protect cardiomyocytes. J Biol Chem, 2013. 288(33): p. 23798-806. The thesis author has minor experimental contributions to this paper.

DISCUSSION

Mitochondria play a significant role in energy production[8, 9] as well as cell death under stress conditions[4, 14, 15, 41]. There is increasing evidence that mitophagy confers cellular protection by eliminating damaged mitochondria and providing a source of energy and nutrients[35, 39, 42]. Although regulation of mitophagy has recently been extensively studied, the molecular mechanisms and signaling pathways involved in this process remain unsolved. We demonstrate here that forced dissociation of hexokinase-II (HK-II) from mitochondria induces Parkin recruitment to mitochondria to elicit subsequent mitophagy. Interestingly our results show that HK-II binds to mitofusin2 (mfn2), a postulated Parkin receptor at mitochondria[38].

HK-II dissociation and 15G

It is now well established that a significant portion of HK-II binds to mitochondria through its N-terminus[22]. We and others have previously demonstrated that the N-terminus deletion mutant of HK-II is unable to bind to mitochondria (Figure 2). A short HK-II N-terminus derived peptide attached to cell-permeable sequences (antennapedia or TAT) has been used as a HK-II dissociating peptide to determine the role of mitochondrial HK-II[29, 30]. We generated an adenovirus encoding 15 amino acids from the N-terminus of HK-II fused with GFP (15G) and demonstrated that the 15G peptide localizes at

mitochondria as shown by co-localization of GFP and TMRE fluorescence (Figure 3). Furthermore, expression of 15G significantly decreased mitochondrial HK-II binding in a dose dependent manner (Figure 4).

15G induces mitophagy.

The current study demonstrates that expression of 15G, at a dose that dissociates mitochondrial HK-II by 40% (150 MOI), also induces robust ubiquitination of mitochondrial proteins (Figure 8A). The levels of VDAC and COX-IV were also markedly reduced by 15G while neither nuclear (Lamin A/C) nor cytosolic (GAPDH) proteins were affected (Figure 5). These findings suggest that significant dissociation of HK-II from mitochondria elicits mitochondrial ubiquitination and subsequent mitophagy. Of additional interest, VDAC degradation was blocked by a lysosomal inhibitor, further indicative of mitophagy (Figure 6).

HK-II dissociation induces Parkin translocation

Parkin translocation to mitochondria has been established as a critical mediator of mitophagy[36, 43], leading to ubiquitination of various mitochondrial targets and facilitating the recruitment of adapter proteins to the mitochondrion. This translocation has been shown to occur in response to simulated ischemia in NRVMs or cells in which mitochondria are depolarized by treatment with FCCP or CCCP[40]. We confirmed that ischemia induced mitochondrial HK-II dissociation causes an increase in mitochondrial Parkin (data not shown) and

further show that this occurs in response to 15G treatment (Figure 8B). The ability of WT HK-II to overcome mitochondrial accumulation of Parkin induced by 15G (Figure 8C) while having no effect when used alone further supports the role of mitochondrial HK-II dissociation, rather than nonspecific increases in mitochondrial protein binding, in Parkin translocation to the mitochondria and subsequent ubiquitination.

Mitochondrial membrane depolarization has been demonstrated to elicit Parkin translocation to mitochondria through PINK1[36, 43]. However, we have observed that TMRE fluorescence is intact in cells infected with 15G at 150 MOI, undistinguishable from control cells (Roberts and Miyamoto, unpublished observation), suggesting that mitochondrial membrane depolarization is not induced by HK-II dissociation mediated by 15G expression. This is also supported by our finding that 15G expression did not induce mitochondrial accumulation of full-length PINK1, as mitochondrial depolarization stabilizes PINK1 through inhibition of proteasomal degradation (Figure 10). Although high dose of TAT-fusion peptide has previously been reported to induce opening of the mPTP leading to mitochondrial depolarization, our results suggest that mPTP opening and resultant mitochondrial membrane depolarization are not involved in 15G induced mitophagic effects, since treatment of cyclosporine A elicited no changes in 15G induced Parkin translocation to mitochondria, mitochondrial protein ubiquitination (Figure 9A), and VDAC loss (Figure 9B). Taken together, these data suggest that

mitochondrial HK-II dissociation induces mitophagy by a mitochondrial membrane depolarization independent mechanism. This is also supported by our observation that HK-II dissociation is able to further increase ubiquitination of mitochondrial proteins induced by FCCP (Figure 7).

Mitofusin2 and HK-II association

Mitofusin2 (mfn2) is an outer mitochondrial membrane protein that has recently been reported to be a binding partner for Parkin at mitochondria[38]. Remarkably, HK-II is co-immunoprecipitated with mfn2 in cells overexpressing HK-II, and this association was significantly reduced by simulated ischemia (Figure 11A). Most importantly, we showed that 15G expression decreased the interaction between endogenous HK-II and mfn2 (Figure 11B). These data suggest that mfn2 serves as a mitochondrial binding partner for HK-II. Ischemia induced mitochondrial HK-II dissociation has been suggested to be due to accumulation of glucose 6-phosphate, a product of HK-II's catalytic activity under ischemic conditions[28]. Indeed, we observed that endogenous HK-II binds to mfn2 in NRVMs and that this association is significantly decreased in response to simulated ischemia (Figure 11C, D). These results implicate mfn2 as a novel mitochondrial binding site of HK-II which is disrupted by ischemia, vacating mfn2 for Parkin binding. These data overall provide considerable insight into the mechanisms behind mitochondrial HK-II dissociation induced mitophagy.

Future studies

More research still needs to be done to further explore HK-II involvement in the regulation of mitophagy. While we show a strong correlation between Parkin translocation to mitochondria and ubiquitination of mitochondrial proteins, Parkin knockdown experiments would be important to establish that the observed ubiquitination is indeed due to Parkin activity. Mfn2 has been shown to be necessary for PINK1 and Parkin mediated mitophagy, but the exact role it plays in interacting with HK-II requires further investigation. Potential knockdown of mfn2 may provide insight into the functional relevance of HK-II binding to mfn2. Finally, we are also aiming to move this project into an in-vivo mouse model and are in the process of generating transgenic mice with inducible cardiac expression of the 15G peptide.

Increase in mitochondrial HK-II binding has been established to confer mitochondrial protection against acute oxidative stress[5, 22] and we have previously reported that Akt phosphorylation of HK-II leads to increase in mitochondrial HK-II, thus contributing to Akt mediated preservation of mitochondrial integrity against ROS or Ca^{2+} overload[24]. In this study, we demonstrate that decreasing mitochondrial HK-II below basal levels elicits Parkin translocation to mitochondria, ubiquitination of mitochondria and subsequent mitophagy. Interestingly, this effect appears to be independent of mitochondrial depolarization. Under sustained ischemia, mitochondrial respiration is inhibited, resulting in mitochondrial depolarization and decreased

ATP production[5, 9], and mitochondrial HK-II binding is also decreased[13, 26]. Our results suggest that dissociation of HK-II from mitochondria during ischemic stress can function as a signal to the induction of mitophagy in concert with the established effect of mitochondrial depolarization. The mitophagic effect of mitochondrial HK-II dissociation could alleviate this ischemic stress by eliminating dysfunctional mitochondria and providing a source of cellular energy and nutrients during sustained metabolic stress. These results suggest that binding of the glycolytic kinase HK-II to mitochondria tightly couples to mitochondrial protection as well as quality control. Better understanding of how HK-II regulates them in the heart may potentially help unlock novel therapeutic options against heart disease in the future.

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