HIF-1 reduces ischaemia–reperfusion injury in the heart by targeting the mitochondrial permeability transition pore

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Aims
Hypoxia-inducible factor-1 (HIF-1) has been reported to promote tolerance against acute myocardial ischaemia–reperfusion injury (IRI). However, the mechanism through which HIF-1 stabilization actually confers this cardioprotection is not clear. We investigated whether HIF-1a stabilization protects the heart against acute IRI by preventing the opening of the mitochondrial permeability transition pore (MPTP) and the potential mechanisms involved.

Methods and results
Stabilization of myocardial HIF-1 was achieved by pharmacological inhibition of prolyl hydroxylase (PHD) domain-containing enzyme using GSK360A or using cardiac-specific ablation of von Hippel–Lindau protein (VHLfl/fl) in mice. Treatment of HL-1 cardiac cells with GSK360A stabilized HIF-1, increased the expression of HIF-1 target genes pyruvate dehydrogenase kinase-1 (PDK1) and hexokinase II (HKII), and reprogrammed cell metabolism to aerobic glycolysis, thereby resulting in the production of less mitochondrial oxidative stress during IRI, and less MPTP opening, effects which were shown to be dependent on HKII. These findings were further confirmed when HIF-1 stabilization in the rat and murine heart resulted in smaller myocardial infarct sizes (both in vivo and ex vivo), decreased mitochondrial oxidative stress, and inhibited MPTP opening following IRI, effects which were also found to be dependent on HKII.

Conclusions
We have demonstrated that acute HIF-1a stabilization using either a pharmacological or genetic approach protected the heart against acute IRI by promoting aerobic glycolysis, decreasing mitochondrial oxidative stress, activating HKII, and inhibiting MPTP opening.

Keywords
Energy metabolism • Hypoxia-inducible factor • Ischaemia • Mitochondria • Reperfusion

1. Introduction
The mitochondrion has emerged as a critical mediator of cell death when the heart is subjected to ischaemia–reperfusion injury (IRI). During reperfusion, calcium overloading, elevated levels of reactive oxygen species (ROS), and abrupt restoration of pH, all act in concert to induce mitochondrial dysfunction leading to the formation of a non-selective mitochondrial channel, termed the mitochondrial permeability transition pore (MPTP).1 The opening of the MPTP then leads to loss of mitochondrial membrane potential, leakage of mitochondrial proapoptotic factors, and ATP depletion, ultimately leading to cell death.2,3 Inhibition of MPTP opening at this time has been demonstrated to be an effective strategy for protecting the heart against the detrimental effects of IRI.4–6

A seminal study, by Murry et al.7 in 1986, demonstrated that myocardial infarct size can significantly be reduced by subjecting the heart to one or more episodes of non-lethal myocardial ischaemia and reperfusion prior to the index ischaemia, a phenomenon termed ischaemic preconditioning (IPC). We have linked suppression of MPTP opening at the onset of reperfusion with the protection afforded by IPC4 and over the years, the prevention of MPTP opening has remained relevant in...
the quest of improving cell survival after an ischemic episode, a strategy that has not been proved to affect clinical outcomes in patients.

Over the past decade, the transcriptional complex hypoxia-inducible factor 1 (HIF-1) has emerged as a key regulator of the molecular hypoxic response. HIF-1 is a critical oxygen-sensitive transcription factor that orchestrates the body’s protective response to hypoxia through the transcriptional activation of up to 200 genes, some of which are critical to cell survival, and may therefore be important in the adaptation of the heart to tolerate IRI.2 HIF-1 comprises an oxygen-sensitive α-subunit and a constitutively-expressed β-subunit and is rapidly degraded under normoxia and stabilized during low levels of oxygen.9,10 The stabilization of HIF-1α under normoxic conditions using genetic manipulation or PHD inhibitors has been documented in various experimental studies to protect the heart from IRI through the transcriptional activation of known cardioprotective factors and mediators.11–15 In addition, there is emerging evidence that HIF-1α activation may act as a mediator of IPC. Hearts from HIF-1α−/− failed to demonstrate protection against prolonged ischemia despite being subjected to an IPC stimulus, which protected wild-type mice hearts.16 Inhibition of HIF-1α expression through small interfering RNAs also blocked IPC in a separate study.17

The glycolytic enzyme hexokinase (HK), which is also an established downstream target gene of HIF-1, has also been increasingly studied as a main regulator of the MPTP. This is consistent with the role of HK in mitochondrial function and cell death including binding to and regulation of mitochondrial voltage-dependent anion channel and its regulatory mediator of IPC. Hearts from HIF-1α−/− mice show expression of mitochondrial HKII is a critical determinant of infarct size, and a constitutively-expressed HIF-1α subunit to cell survival, and may therefore be important in the adaptation of the heart to tolerate IRI.1 HIF-1α comprises an oxygen-sensitive α-subunit and a constitutively-expressed β-subunit and is rapidly degraded under normoxia and stabilized during low levels of oxygen.9,10 The stabilization of HIF-1α under normoxic conditions using genetic manipulation or PHD inhibitors has been documented in various experimental studies to protect the heart from IRI through the transcriptional activation of known cardioprotective factors and mediators.11–15 In addition, there is emerging evidence that HIF-1α activation may act as a mediator of IPC. Hearts from HIF-1α−/− failed to demonstrate protection against prolonged ischemia despite being subjected to an IPC stimulus, which protected wild-type mice hearts.16 Inhibition of HIF-1α expression through small interfering RNAs also blocked IPC in a separate study.17

The glycolytic enzyme hexokinase (HK), which is also an established downstream target gene of HIF-1, has also been increasingly studied as a main regulator of the MPTP. This is consistent with the role of HK in mitochondrial function and cell death including binding to and regulation of mitochondrial voltage-dependent anion channel and its regulatory role in mitochondrial pore formation.18–22 Of the four HK isozymes, HKI and II are enriched in the heart, and HKII expression levels and localization of mitochondrial HKII, hearts were perfused with HKII-dissociating peptide or control peptide during the last 20 min of stabilization at 200 nM. Adult mouse and rat cardiomyocytes were isolated as described previously.18

### 2.2 In vitro experiments

HL-1 cells were cultured as previously described.29 Where indicated, cells were treated with either DMSO or GSK360A. siRNA experiments were performed 24 h prior to DMSO or GSK360A treatment. Overexpression of HKII was performed by transfecting HL-1 cells with pCMV6-HK2 (Origene) using Lipofectamine LTX with Plus™ reagent (Applied Biosystems). mRNA and protein expression were analysed by real-time PCR and western blot, respectively. Detailed proteomic analysis is described in Supplementary material online. Oxygen consumption was monitored using the fluorescent oxygen probe MitoXpress-Xtra-HS (Lux-cell Biosciences, Ireland) according to manufacturer’s instructions. Fluorescence microscopy was performed to determine resting mitochondrial membrane potential (tetramethylrhodamine, ethyl ester, TMRE), basal mitochondrial ROS (MitoXpress-Xtra-HS) fluorescence, and western blot, respectively. Detailed proteomic analysis is described in Supplementary material online. Oxygen consumption was monitored using the fluorescent oxygen probe MitoXpress-Xtra-HS (Lux-cell Biosciences, Ireland) according to manufacturer’s instructions. Fluorescence microscopy was performed to determine resting mitochondrial membrane potential (tetramethylrhodamine, ethyl ester, TMRE), basal mitochondrial ROS (MitoXpress-Xtra-HS) fluorescence, and western blot, respectively. Detailed proteomic analysis is described in Supplementary material online.

### 2.3 Statistical analysis

The data shown are presented as the mean ± standard error of three or more independent experiments. Differences are considered statistically significant at P < 0.05, assessed using Student’s t-test (for paired samples) or the ANOVA test (for more than two groups) followed by post hoc analysis using Tukey’s test.
Figure 1 GSK360A treatment leads to normoxic stabilization of HIF-1 and up-regulation of its target genes. Gene expression of selected HIF-1 target genes was determined following varying durations of GSK360A treatment at two different concentrations. *P < 0.05 vs. DMSO-treated cells at time 0 (N = 5) (A). Densitometry analysis of immunoblotting for HIF-1 and HKII following 8 h of GSK360A treatment at 50 μM. α-Tubulin was used as a loading control for whole cell lysate; COX IV was used as a loading control for mitochondrial fraction while lactate dehydrogenase (LDH) served as a negative control for mitochondrial fraction. Dimethylhyaloxylgycine (DMOG) was used as a positive control for HIF-1 stabilization. Blots shown are representative of four individual experiments. *P < 0.05 vs. DMSO-treated cells (N = 4) (B). Knockdown of HIF-1 prevented up-regulation of its target genes despite GSK360A treatment. #P < 0.05 vs. DMSO-treated cells. *P < 0.05 vs. GSK360A-treated cells transfected with scrambled-siRNA (N = 5) (C).
performed on HL-1 cells treated for 8 h with 50 μM of GSK360A as this dosing regimen promoted an increase in the glycolytic-related genes PDK1 and HKII. Immunoblotting of whole cell lysate further confirmed an increase in HIF-1α protein levels, and this corresponded with an increase in HKII in the purified mitochondrial fraction (Figure 1B). Importantly, treatment of HL-1 cells with GSK360A failed to induce activation of HIF-1 target genes except heme oxygenase 1 (HO-1), when cells were pretreated with siRNA-targeting HIF-1α (Figure 1C and see Supplementary material online, Figure S1). These results indicate that treatment of HL-1 cells with GSK360A stabilized HIF-1α under normoxic conditions resulting in the transcription of downstream genes.

3.2 GSK360A treatment of HL-1 cells promoted aerobic glycolysis

To explore the molecular pathways regulated by normoxic HIF-1 stabilization, we subjected whole cell extracts harvested from cells treated with either DMSO or GSK360A to relative quantitative proteomic analysis (Figure 2A). Using a fold change cut-off of ≥2, 49 proteins were found to be down-regulated by GSK360A treatment, whereas 81 proteins were shown to be up-regulated (see Supplementary material online, Tables SI and SII). Gene ontology analysis using the PantherDB database revealed an enrichment in the percentage of proteins involved in metabolic pathways. Representative time-resolved fluorometry measurement of oxygen consumption in HL-1 cells treated with or without GSK360A at 37°C over 4 h (C). O2 consumption of GSK360A-treated vs. control cells for each time point was measured by linear regression in order to calculate the slopes. The effects of HIF-1 or HKII inhibition on O2 consumption were also determined. O2 consumption rate was normalized to protein content. *P < 0.05 vs. DMSO-treated cells at each indicated time duration (N = 4) (D).
in the metabolic processes and the generation of precursor metabolites and energy (Figure 2B), which is in accordance with previous studies implicating metabolic reprogramming away from oxidative phosphorylation (OXPHOS) towards aerobic glycolysis with HIF-1 stabilization.31,32 Accordingly, we observed lower oxygen utilization in HL-1 cells treated with GSK360A compared with DMSO-treated cells when measured using a fluorescent oxygen probe (representative image of a single experiment shown in Figure 2C). Relative oxygen consumption of GSK360A-treated cells was also significantly lower when measured at different time intervals (Figure 2D). This metabolic switch was dependent on HIF-1, because HL-1 cells depleted of HIF-1 failed to demonstrate reduced oxygen consumption following GSK360A treatment (Figure 2D). The role of HKII as a downstream effector of HIF-1-mediated metabolic switch was also confirmed by knockdown of HKII, which partially prevented HL-1 cells from decreasing oxygen consumption despite GSK360A treatment (Figure 2D and see Supplementary material online, Figure S2).

To further characterize the metabolic switch from OXPHOS to aerobic glycolysis mediated by normoxic HIF-1 stabilization, we measured mitochondrial membrane potential (Δψm) and mitochondrial ROS production under basal conditions. Cells treated with GSK360A had a significantly lower Δψm as measured by reduced TMRE fluorescence, compared with DMSO-treated cells alone (Figure 3A). As mitochondrial ROS are generated during OXPHOS, we also investigated whether GSK360A treatment would decrease mitochondrial ROS levels. We examined mitochondrial ROS in DMSO-treated and GSK360A-treated cells using MTR CM-h2XROS, a mitochondrial specific dye that fluoresces when oxidized by ROS. Under basal conditions, GSK360A-treated cells showed significantly lower levels of mitochondrial ROS compared with vehicle-treated cells (Figure 3B). Consistent with the decrease in OXPHOS, GSK360A also decreased the expression of antioxidant genes (Figure 3C) and conversely led to an increase in intracellular lactate levels (Figure 3D). Previous studies have reported an increase in glycogen levels when cells were exposed to hypoxia or hypoxia mimetics.33 However, HL-1 cells treated with GSK360A for 8 h had comparable intracellular glycogen compared with DMSO-treated cells, indicating that short-term stabilization of HIF-1α in our study does not lead to a significant accumulation of glycogen (Figure 3E and see Supplementary material online, Figure S3).

3.3 GSK360A treatment protected HL-1 cells against acute IRI—a role of HKII
We next investigated whether HIF-1-induced reprogramming of basal metabolism is relevant for protection against acute IRI, and whether this effect is mediated by the HIF-1 downstream target gene, HKII. HL-1 cells were pretreated with either DMSO or GSK360A before being subjected to in vitro simulated IRI. Cell death was then determined by PI exclusion, and mitochondrial HKII levels were measured by immunoblotting. As evidenced in Figure 4A, cells treated with GSK360A had significantly lower cell death compared with DMSO-treated cells, and this was associated with increased mitochondrial HKII levels (Figure 4B). To investigate the role of HKII in mediating this protective effect, cells treated with siRNA against HIF-1 or HKII were subjected to a similar protocol. Indeed, knockdown of HKII completely abolished the protective effects of GSK360A, leading to increased cell death compared with DMSO-treated cells while knockdown of HKII abolished the protective effects of GSK360A (Figure 4A). In contrast, HL-1 cells overexpressing HKII were found to have increased tolerance against in vitro IRI (see Supplementary material online, Figure S4).

Immunoblotting of HKII revealed that knockdown of either HIF-1 or HKII resulted in decreased HKII levels in isolated mitochondrial fraction, demonstrating the protective effects of HIF-1 against IRI were partially dependent on mitochondrial HKII (Figure 4B).

To further investigate the mechanism by which IRI is attenuated by normoxic HIF-1 stabilization, we evaluated mitochondrial function in HL-1 cells during reperfusion focusing on oxidative stress and MPTP opening, both of which are important mediators of cell death at this time and have been reported to be modulated by mitochondrial HKII. Consistent with lower cell death, GSK360A-treated cells exhibited less mitochondrial oxidative stress after IRI as indicated by reduced fluorescence of MitoSOX Red, which fluoresces upon oxidation by mitochondrial superoxide (Figure 4C). This was accompanied by inhibited MPTP opening as indicated by improved retention of mitochondrial-entrapped calcine-AM following reperfusion (Figure 4D). In this assay, MPTP opening during reperfusion allows the redistribution of mitochondrial localized calcine to the cytosol where its fluorescence is quenched. In contrast, siRNA-targeting HIF-1 resulted in higher oxidative stress and increased MPTP opening in cells treated with GSK360A, demonstrating its dependence on HIF-1 in mediating cardioprotection against IRI (Figure 4C and D). The role of HKII in HIF-1-mediated cardioprotection was also demonstrated by experiments in which cells pretreated with a siRNA-targeting HKII followed by GSK360A treatment failed to limit production of oxidative stress and to prevent MPTP opening following IRI (Figure 4C and D). MPTP opening was, however, inhibited following in vitro IRI in HL-1 cells overexpressing HKII (see Supplementary material online, Figure S4), providing further proof that HKII is an essential regulator of MPTP. To further interrogate our hypothesis that HIF-1/HKII confers protection through regulation of excessive mitochondrial ROS which limits MPTP opening during early reperfusion, we attempted to suppress mitochondrial ROS formation through knockdown of ROS modulator 1 (ROMO1), which was recently identified as a major source of ROS originating from the mitochondrial electron transport chain (ETC; see Supplementary material online, Figure S5). We observed that inhibition of ROMO1 resulted in HL-1 cells generating less ROS following IRI, even when HIF-1 or HKII was suppressed (Figure 4C). Importantly, this marked reduction of oxidative stress increased HL-1 cells’ resistance to IRI despite having either HIF-1 or HKII suppressed (Figure 4A) and similarly, these cells had lesser MPTP opening following IRI (Figure 4D) proving that reduction of excessive mitochondrial ROS by HIF-1/HKII is crucial for maintenance of mitochondrial integrity.

3.4 GSK360A treatment protected the rat heart against acute IRI—a role of HKII
Having demonstrated in HL-1 cells the importance of the HIF-1/HKII axis in mediating resistance against IRI by limiting ROS and inhibiting MPTP opening, we investigated this protective pathway in Langendorff-perfused adult rat hearts subjected to acute IRI. In vivo pretreatment of rats with GSK360A, administered 4 h earlier by oral gavage, resulted in a significant increase in myocardial HIF-1α stabilization as assessed by immunostaining (Figure 5A), and qPCR of HIF-1 target genes (see Supplementary material online, Figure S6). Although there was no difference in area at risk (AAR) in these excised hearts compared with DMSO-treated hearts following regional LAD occlusion and reperfusion (Figure 5B), GSK360A treatment significantly reduced myocardial infarct size compared with control animals (Figure 5C and see Supplementary material online, Figure S7). To investigate the functional
GSK360A-induced aerobic glycolysis is associated with a decreased use of mitochondrial electron transport chain in HL-1 cells. Basal mitochondrial membrane potential following GSK360A treatment was measured using TMRE at 37°C. *P < 0.05 vs. DMSO-treated cells (N = 4) (A). Basal mitochondrial ROS following GSK360A treatment was measured using Mitotracker Red (MTR) CM-H2XRos at 37°C. *P < 0.05 vs. DMSO-treated cells (N = 4) (B). Aerobic glycolysis following GSK360A treatment is associated with a decreased expression of antioxidant-encoding genes. *P < 0.05 vs. DMSO-treated cells (N = 5) (C). Measurement of intracellular lactate production was performed following GSK360A treatment. *P < 0.05 vs. DMSO-treated cells (N = 4) (D). Quantitation of intracellular glycogen production was performed following GSK360A and expressed as µg per 10^6 cells. *P < 0.05 vs. DMSO-treated cells (N = 4) (E).
importance of mitochondria–HKII association underlying HIF-1-induced cardioprotection, we used a synthetic peptide that contains a cellular uptake motif and the mitochondrial-binding motif of HKII that has been shown to acutely dislodge HKII from mitochondria.34,35 Isolated hearts from DMSO- or GSK360A-treated rats were perfused with this HKII peptide during the initial stabilization period, before being subjected to regional LAD occlusion. Treatment with this HKII peptide decreased the association of HKII with mitochondria following reperfusion (Figure 5D) and prevented the limitation of myocardial infarct size afforded by GSK360A in excised hearts (Figure 5C and see Supplementary material online, Figure S7). In accordance with the earlier results observed in HL-1 cells, hearts with smaller myocardial infarct size had lesser oxidative stress as assessed by protein carbonylation levels (Figure 5E) and higher mitochondrial NAD$^+$ levels indicating lesser mitochondrial damage (Figure 5F). In contrast, treatment of these hearts with HKII peptide reversed the protective effects of GSK360A on
Figure 5 GSK360A protects rat hearts against IRI through preservation of mitochondrial integrity. Adult rats were orally gavaged with GSK360A 4 h prior to sacrifice. Immunostaining of HIF-1 in isolated hearts. Red arrows show nuclear localization of HIF-1 (A). Measurement of myocardial ischaemic AAR, as determined by Evans blue dye (unstained by blue dye) (B), and infarct area [unstained by triphenyltetrazolium chloride (TTC) expressed as a percentage of AAR] (C) following IRI with or without GSK360A treatment. *P < 0.05 vs. DMSO-treated hearts subjected to IRI (N = 6). Representative immunoblots and densitometry quantification of HKII in purified mitochondrial fractions collected from different treatment groups of hearts post-IRI were performed. COX IV was used as a marker for mitochondria and LDH was used as a negative control. *P < 0.05 (N = 4) (D). Protein oxidation as a surrogate marker of oxidative stress (E) and mitochondrial NAD+ (as a marker of mitochondrial health) (F) following IRI were also measured. *P < 0.05 vs. sham animals, #P < 0.05 vs. DMSO-treated hearts subjected to IRI (N = 6). Infarct area in rats pretreated with either DMSO or GSK360A before being subjected to in vivo IRI was measured. **P < 0.05 vs. DMSO-treated rats (N = 8).
mitochondrial function, suggesting that HKII is an important down-stream effector of HIF-1 in limiting oxidative stress and preventing MPTP opening following IRI (Figure 5E and F).

Importantly, the cardioprotective effects of GSK360A were also evident in rats subjected to in vivo IRI. Rats pretreated with GSK360A or DMSO 4 h prior to surgery as described above were subjected to 30 min of ischaemia followed by 24 h of reperfusion. While hearts from both groups had comparable AAR, GSK360A-treated rat hearts had significantly smaller infarct size compared with controls which is consistent with our ex vivo data (Figure 6A and B). Additionally, GSK360A treatment did not significantly affect systolic blood pressure and heart rate, compared with control rats when measured before

![Figure 6](https://example.com/figure6.png)

**Figure 6** Cardioprotective effects of GSK360A in rats and isolated adult rat cardiomyocytes undergoing IRI. Adult rats orally gavaged with either DMSO or GSK360A for 4 h were subjected to in vivo IRI. Twenty-four hours later, rats were sacrificed and hearts were excised for determination of AAR (A) and infarct size (B). *P < 0.05 vs. DMSO-treated rats (N = 8). Effects of GSK360A treatment in isolated adult rat cardiomyocytes on ROS production (C) and MPTP opening (D and E) were determined by MitoSOX, mitochondrial swelling assay, and calcium retention capacity, respectively. The involvement of HIF-1 and HKII were also assessed by inclusion of NSC-134754 (HIF-1 inhibitor) and a HKII-dislodging peptide. White bars indicate normoxic control and black bars indicate samples subjected to IRI. *P < 0.05 vs. DMSO-treated cells in normoxia (N = 6).
and after surgery (see Supplementary material online, Table SIII). To extend the results obtained from intact hearts and whole rats to a subcellular level, we isolated cardiac mitochondria from adult rat cardiomyocytes pretreated with 50 μM of GSK360A for 4 h (see Supplementary material online, Figure S8) before being subjected to IRI. This treatment regime was found to induce a glycolytic switch in adult rat cardiomyocytes similar to that seen in HL-1 cells as indicated by increased glucose analogue uptake as well as decreased oxygen consumption (see Supplementary material online, Figure S9). We subsequently measured production of oxidative stress in these mitochondria using MitoSOX Red, and assessed MPTP opening by measuring calcium retention capacity as well as mitochondrial swelling assay. Consistent with earlier results, mitochondria isolated from cardiomyocytes treated with GSK360A generated significantly lesser superoxide compared with controls, and this reduction was only abolished upon pretreatment with a HIF-1 inhibitor, NSC-134754, or HKII peptide (Figure 6C). Simulated IRI-induced decreases in absorbance reflecting MPTP opening were also significantly inhibited in GSK360A-treated isolated mitochondria compared with DMSO, upon which was abolished in NSC-134754 or HKII peptide-treated isolated mitochondria (Figure 6D), suggesting the importance of both HIF-1 and HKII on mitochondria by reducing oxidative stress thus preventing simulated IRI-induced MPTP opening. Notably, colocalization staining of intact adult rat cardiomyocytes for HKII and COX IV, a mitochondrial marker, revealed that the extent of mitochondrial integrity correlates directly with the binding of HKII to mitochondria (see Supplementary material online, Figure S10), further confirming the importance of mitochondrial HKII in HIF-1-mediated cardioprotection. Similarly, our measurements showed that pretreatment with GSK360A resulted in a decreased sensitivity to Ca^{2+}-induced MPTP opening following IRI compared with untreated cells. Crucially, this protective effect was abrogated when cells were also treated with NSC-134754 or HKII peptide further confirming the importance of both HIF-1 and HKII (Figure 6E). Treatment of adult cardiomyocytes with MitoTEMPo, a mitochondria-targeted antioxidant, successfully reversed the excessive amount of ROS and prevented MPTP opening induced by HKII dislocation (see Supplementary material online, Figure S11) which is in accordance with our earlier observations in HL-1 cells, suggesting that modulation of ROS production by HIF-1/HKII is critical for preventing mitochondrial dysfunction.

3.5 Stabilizing myocardial HIF-1 by genetic ablation of cardiac VHL protects the murine heart against in vivo IRI

Finally, we used cardiac-specific ablation of Von Hippel–Lindau tumor suppressor protein (VHL) as a genetic model to stabilize myocardial HIF-1 to ensure that the aforementioned protective effects were specific to HIF-1 stabilization and not due to PHD inhibition. The schematic diagram of the crossing is shown in Figure 7A. Treatment of VHLfl/fl, α-MHC-Cre mice, with 5 days of tamoxifen resulted in significant inactivation of cardiac VHL and stabilization of HIF-1α as determined by western blotting (Figure 7B). Following vehicle or tamoxifen treatment, mice were subjected to in vivo myocardial IRI. The AAR expressed as a percentage of the left ventricular volume was comparable between all treatment groups (Figure 7C). However, following a lethal episode of IRI, the genetic inactivation of VHL significantly reduced the myocardial infarct size to AAR ratio compared with control animals (Figure 7D). To allow for a more thorough characterization, adult cardiomyocytes were also isolated from these mice following treatment with either vehicle or tamoxifen and subjected to IRI. As shown in Figure 6E, the percentage of cell death following IRI was reduced in adult murine cardiomyocytes in which VHL was inactivated. The improved cell survival following IRI was completely abrogated by pretreatment with the direct HIF-1α inhibitor, NSC-134754 (Figure 7E). Compared with cardiomyocytes isolated from vehicle-treated mice, cardiomyocytes isolated from tamoxifen-treated mice generated lesser superoxide (Figure 7F), leading to inhibited MPTP opening (Figure 7G).

4. Discussion

A number of experimental studies have reported that normoxic HIF-1α stabilization using non-specific pharmacological PHD inhibitors (such as cobalt chloride and desferrioxamine) or genetic manipulation of HIF-1α or PHD can protect the heart from acute IRI (reviewed in Ong and Hau senlooy). In the majority of these studies, acute HIF-1 stabilization resulted in a delayed cardioprotective effect 12–24 h later through the transcription and activation of known cardioprotective mediators, such as erythropoietin, HO-1, and inducible nitric oxide synthase. However, it has been recently shown that acute HIF-1α stabilization may confer early cardioprotection, and it has also been suggested that HIF-1 may mediate the beneficial effects of classical IPC, in which one or more cycles of brief non-lethal ischaemia and reperfusion protects against a subsequent acute myocardial infarction. However, despite all these studies, the actual mechanism through which HIF-1α stabilization protects the heart against IRI is unclear. In this study, we present evidence for a previously undescribed role of HIF-1 in protecting against IRI through the inhibition of MPTP opening. We demonstrated that normoxic stabilization of HIF-1α reprogrammed cell metabolism from OXPHOS to aerobic glycolysis, decreasing the production of mitochondrial oxidative stress during IRI, thereby inhibiting MPTP opening. We also demonstrated the requirement of mitochondrial HKII as a downstream effector of HIF-1 in preventing mitochondrial dysfunction following IRI.

The production of intra-mitochondrial oxidative stress and the subsequent opening of the MPTP are important signs of mitochondrial dysfunction during IRI and are critical determinants of cardiomyocyte death. A recent study demonstrated that HIF-1α stabilization induced by genetic PHD2 ablation in skeletal muscle reprogrammed cell metabolism from aerobic to anaerobic respiration under normoxic conditions, thereby impairing exercise capacity under normoxic conditions, but rendering the skeletal muscle resistant to IRI. A similar effect was seen in neonatal cardiomyocytes with HIF-1 stabilization arising from PHD inhibition promoting anaerobic respiration during IRI. Importantly, in our study, we found that the novel PHD inhibitor, GSK360A, also reprogrammed cell metabolism from OXPHOS to aerobic glycolysis under basal conditions as evidenced by: the activation of glycolytic protein such as PDK1 and HKII, decreased mitochondrial respiration with increased intracellular lactate production, partial mitochondrial membrane depolarization, less mitochondrial ROS production, and decreased levels of antioxidants. This metabolic switch prior to an ischaemic insult leads to inactivity of the mitochondrial ETC, thereby resulting in the production of lesser intra-mitochondrial oxidative stress during myocardial ischaemia such that MPTP opening at the onset of myocardial reperfusion is inhibited.

Analysis of initial gene expression of HL-1 cells demonstrated the potency of the novel specific PHD inhibitor, GSK360A, in stabilizing HIF-1α, leading to up-regulation of several of its downstream target genes including HKII. We were particularly interested in HKII, previously documented to be a direct HIF-1 target, due to several reasons: (i) rate
Figure 7 Genetic stabilization of HIF-1 protects against IRI by attenuating production of oxidative stress hence preventing MPTP opening. Schematic diagram depicting the creation of cardiac-specific VHL^fl/fl, α-MHC-Cre mice (A). Immunoblots for VHL and HIF-1 from transgenic VHL^fl/fl, α-MHC-Cre mice and densitometry quantification from three individual experiments. *P < 0.05 vs. DMSO-treated transgenic mice (B). Measurement of myocardial ischaemic AAR was determined by Evans blue dye (unstained by blue dye) and infarct area (unstained by TTC expressed as a percentage of AAR) following in vivo IRI. *P < 0.05 vs. wild-type tamoxifen-treated mice (N = 6) (C and D). Isolated adult mouse cardiomyocytes were isolated from DMSO (black bars) and tamoxifen-treated (grey bars) transgenic mice and subjected to in vitro IRI. Cell death was determined by PI control. Insulin was included as a positive control for tolerance against ischaemic stress, and NSC-134754 was used as a HIF-1 inhibitor. *P < 0.05 vs. DMSO-treated cardiomyocytes in normoxia. #P < 0.05 vs. DMSO only-treated cardiomyocytes subjected to IRI (N = 6) (E). Measurement of mitochondrial ROS (F) and MPTP opening (G) were also determined in these cardiomyocytes following IRI, MPG, an antioxidant and cyclosporine A (CsA), a known MPTP inhibitor were included. *P < 0.05 vs. DMSO-treated cardiomyocytes in normoxia. #P < 0.05 vs. DMSO only-treated cardiomyocytes subjected to IRI (N = 6).
affording cardioprotection.48 One possible explanation behind these by studies demonstrating the need for ROS in stabilizing HIF-1 and ferroxamine and ethyl-3,4-dihydroxybenzoate up-regulates HIF-1 and with previous findings, showing that the use of iron chelators such as des-
consequence of lesser ETC activity. This was, however, in contrast observed decreased production of ROS in HL-1 cells at basal condition following normoxic HIF-1 stabilization which we postulated to be a one would expect to decrease the production of oxidative stress during IRI, which is beneficial as excessive ROS is known to induce MPTP opening.47 In keeping with this interpretation, we found that GS360A treatment afforded HL-1 cells better tolerance against IRI by limiting generation of oxidative stress during reperfusion hence preventing MPTP opening. The beneficial effects of HIF-1 on limiting production of oxidative stress and inhibiting MPTP opening were also observed in intact rat hearts and isolated mitochondria crucially. Of note, we observed decreased production of ROS in HL-1 cells at basal condition following normoxic HIF-1 stabilization which we postulated to be a consequence of lesser ETC activity. This was, however, in contrast with previous findings, showing that the use of iron chelators such as desferoxamine and ethyl-3,4-dihydroxybenzoate up-regulates HIF-1 and increases generation of mitochondrial ROS which is further complicated by studies demonstrating the need for ROS in stabilizing HIF-1 and affording cardioprotection.48 One possible explanation behind these discrepancies might be due to the pharmacological mode of actions employed by these drugs. While GS360A inhibits PHD specifically by competing with the alpha-ketoglutarate site, the aforementioned iron chelators have been reported to also mediate redox reactions such as prevention of iron cycling. Desferoxamine in the past have been reported to actually increase production of hydroxyl radicals and exert a pro-oxidant and cytotoxic action49 and as such, caution should be exercised when evaluating potential efficacy of HIF-1 activators for future studies.

Having shown that HIF-1 induces a metabolic switch which prevents lethal ROS from priming MPTP opening; we further demonstrated that this is mediated partially through mitochondrial HKII. Compared with controls, GS360A treatment was found to enhance mitochondrial HKII levels both before ischaemia and during reperfusion period, which coincidentally correlates with the degree of protection against cell death or infarct size as measured in our in vitro and in vivo studies. Interestingly, previous studies demonstrated that IPC also invokes a bi-phasic activation of mitochondrial HKII pre- and post-ischaemia as seen here highlighting the similarities between HIF-1 activation and IPC.25 Several mechanisms through which mitochondrial HKII can restrain cellular damage have been proposed.50 Overexpression of HKII leading to mitochondrial localization can lower the level of mitochondrial ROS, which protect cells against oxidative stress-induced cell death. Furthermore, association of HKII with mitochondria has been suggested to inhibit cell death by preventing the activation of the MPTP or by abrogating the activity of the proapoptotic Bcl-2 family proteins. Consistent with these reports, we have shown here that increased mitochondrial HKII lowered production of oxidative stress and inhibited MPTP opening. Association of mitochondria—HKII also reduced cell death and myocardial infarct size following IRI, and crucially these protective effects were reversed when displacement of mitochondrial HKII was forced pharmacologically.

To ensure that all the aforementioned effects were due to HIF-1α sta-
bilization and not due to PHD inhibition, we also employed a novel method to investigate the cardioprotective effects of HIF-1α stabilization using the genetic inactivation of VHL in mice where Cre expression was limited to cardiac tissue only and could be controlled temporally. We found that 5 days of tamoxifen treatment was enough to down-regulate VHL and stabilize HIF-1α. This short-term genetic inactivation of VHL resulted in a large reduction in myocardial infarct size following in vivo IRI. Importantly, adult mouse cardiomyocytes isolated from these mice were also resistant to IRI by reducing oxidative stress, thus prevent-
ing MPTP opening.

Because of the requirement to administer the treatment prior to the index ischaemia, it cannot be implemented into future treatment for patients presenting with an acute myocardial infarction, and therefore unlikely to reduce the burden of ischaemic heart disease mortality worldwide. However, GS360A can potentially be used as a cardioprotec-
tive therapy for patients in which the index myocardial ischaemic event can be reliably predicted such as planned cardiac bypass surgery, percutaneous coronary intervention, or cardiac transplantation, although further optimization is anticipated as this strategy has yet to show any clinically relevant benefits to date.

In conclusion, this is the first study to emphasize the role of HIF-1 in protecting the heart against acute IRI by inhibiting MPTP opening. Furthermore, we show that HIF-1 stabilization inhibits MPTP opening by reprogramming basal cell metabolism from OXPHOS to aerobic glycolysis, such that less mitochondrial oxidative stress is produced during IRI and MPTP opening is inhibited. Finally, we implicated the HIF-1 downstream target gene, HKII, as a mediator of HIF-1 cardioprotection in a similar manner to that of IPC.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

**Conflict of interest:** P.H.M. is a director of ReOx Ltd.

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