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Review

Hypoxia-inducible factor 1 signalling, metabolism and its therapeutic potential in cardiovascular disease☆

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ABSTRACT

Cardiovascular disease (CVD) accounts for the largest number of deaths worldwide, necessitating the development of novel treatments and prevention strategies. Given the huge energy demands placed on the heart, it is not surprising that changes in energy metabolism play a key role in the development of cardiac dysfunction in CVD. A reduction in oxygen delivery to the heart, hypoxia, is sensed and responded to by the hypoxia-inducible factor (HIF) and its family of proteins, by regulating the oxygen-dependent signalling cascade and subsequent response. Hypoxia is one of the main drivers of metabolic change in ischaemic disease and myocardial infarction, and we therefore suggest that HIF may be an attractive therapeutic target. In this review, we assess cardiac energy metabolism in health and disease, and how these can be regulated by HIF-1α activation. We then present an overview of research in the field of hypoxia-mimetic drugs recently developed in other treatment fields, which provide insight into the potential of systemic HIF-1α activation therapy for treating the heart.

1. Introduction

The healthy adult heart extracts between 8 and 70 ml of oxygen per minute per 100 g from the circulation, predominantly to power oxidative metabolism and energy generation. Oxygen is the terminal electron acceptor for oxidative phosphorylation, a process estimated to contribute 90% to cardiac energy generation. Under these normoxic conditions, optimal metabolism drives optimal function of the heart, with the average human adult heart pumping over 7000 l of blood per day.

Hypoxia, a decrease in oxygen availability, has the capacity to impact on metabolism, morphology and function of the heart. Hypoxia can arise as a consequence of both physiological and pathological stimuli. From a physiological perspective, the foetal heart develops in a hypoxic environment, undergoing a hypoxic to normoxic transition at birth. The adult heart can equally be challenged physiologically by hypoxia, whether due to altitude exposure or in response to high intensity exercise. These stimuli induce whole body responses that can have a direct primary effect in the heart, as well as a secondary impact on the heart. From a pathological perspective, the heart may encounter hypoxia due to diseases of cardiac origin as well as diseases affecting peripheral tissues. Examples of the latter include situations as diverse as anaemia, lung diseases and sleep apnoea, which can all reduce oxygen delivery to the heart [1,2]. This review will focus on cardiovascular diseases that directly change oxygen availability for the cardiomyocytes, which induce regions of hypoxia within the myocardium, including myocardial infarction (MI) and heart failure (HF).

Abbreviations: 2-OG, 2-oxoglutarate; ADP, adenosine diphosphate; AMPK, AMP-activated protein kinase; ARNT, aryl hydrocarbon receptor nuclear translocator; ATP, adenosine triphosphate; C-TAD, C-terminal activation domain; CAT, carnitine acyltranslocase; CBP, CREB binding protein; CODD, C-terminal oxygen-dependent degradation domain; CPT, carnitine palmitoyltransferase; DMOG, dimethyloxalyl glycine; EPO, erythropoietin; ET1, endothelin 1; FABPm, fatty acid binding protein (membrane); FADH, flavin adenine dinucleotide H^+; FAT, fatty acid translocase; FATP, fatty acid transporter protein; FIH, factor inhibiting HIF; GLUT, glucose transporter; HBS, HIF-binding sites; HIF, hypoxia-inducible factor; HLH, helix-loop-helix; HO1, haem oxygenase 1; HRE, HIF response elements; IFM, interfibrillar mitochondria; KO, knock-out; LCFA, long-chain fatty acids; LDH, lactate dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; MCT, monocarboxylate transporter; N-TAD, N-terminal activation domain; NADH, nicotinamide adenine dinucleotide H^+; NEFA, non-esterified fatty acid; NODD, N-terminal oxygen-dependent degradation domain; PAS, Per-ARNT-Sim; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PHD, prolyl hydroxylase domain; PPAR, peroxisome proliferator-activated receptor; pVHL, von Hippel–Lindau tumor suppressor; ROS, reactive oxygen species; SSM, subsarcolemmal mitochondria; UCP, uncoupling protein; VEGF, vascular endothelial growth factor

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The cellular mechanism that has evolved in response to hypoxia is highly evolutionarily conserved, present in organisms as diverse as Cnidarian sea anemones, cartilaginous fish and humans [3]. The major transcription factors responding to hypoxia and adapting the cell accordingly are the hypoxia-inducible factors (HIF). The hypoxia-inducible factors (HIFs) are a family of obligate heterodimeric transcription factors that play a key role in mediating cellular adaptation to hypoxia [4], first identified in erythropoietin-producing hepatoma cells [5]. Broadly, HIFs target genes adapt the cell to this new lower oxygen environment, by downregulating processes that consume oxygen, upregulating anaerobic processes, and restoring normal oxygen delivery. In this review we will discuss the cellular mechanisms that sense and respond to hypoxia, the impact they have on metabolism, how this manifests in cardiovascular diseases, and the therapeutic potential for targeting hypoxia-inducible factor signalling in cardiac disease.

2. Hypoxia signalling

2.1. Hypoxia-inducible factor

HIFs are heterodimers composed of an unstable α-subunit (HIFα), of which levels and activity are tightly and acutely controlled by cellular oxygen concentrations, and a stable β-subunit (HIFβ), which is a constitutively-expressed aryl hydrocarbon receptor nuclear translocator (ARNT) [6,7]. In hypoxia, protein levels of HIF-α are stabilized, and the protein travels to the nucleus to dimerise with its HIF-β partner (Fig. 1). The resultant HIFα/β complex binds to specific hypoxia response element (HRE) promoter regions of target genes, and regulates their expression [8].

Three HIF-α isoforms exist in humans: HIF-1α, HIF-2α and HIF-3α. Of these, the first two are better characterised, forming the active transcription factors HIF-1 and HIF-2 when bound to HIF-β subunits. While they share similar protein structures, the different isoforms serve distinct physiological roles, with unique target genes and mechanisms of action [9]. It has been shown in some cell lines that HIF-1 primarily mediates the acute hypoxic response (< 24 h), whereas HIF-2 drives the response during chronic hypoxic challenge (> 24 h) [10,11]. Thus the two isoforms can interact via a complex HIF 'switch' mechanism to regulate the cellular response to hypoxia, each with specific temporal and functional roles.

Both HIF-α and -β-subunits are bHLH/PAS transcription factors, with each comprising a bHLH (basic helix-loop-helix) motif and two hydrophobic PAS (Per-ARNT-Sim) motifs (PAS-A and PAS-B), which allow heterodimerisation, nuclear localization and DNA binding [7,12]. When HIF-1α and -β-subunits bind and stabilise, this recruits transcriptional co-activators p300 and CREB-binding protein (CBP) [13,14] (Fig. 1). This is an additional oxygen-dependent interaction, which can be prevented by factor inhibiting HIF (FIH) [14]. HIFα contains a C-terminal transactivation domain (C-TAD) that recruits coactivator proteins to create a transcriptional complex (although some HIF-3α splice variants have been described which lack a transactivation domain, instead possessing other inhibitory domains) [15,16]. In addition, HIF-α subunits contain a N-terminus transcriptional activation domain (N-TAD), as well as C- and N-terminal oxygen-dependent degradation domains (CODD and NODD, respectively), which allow oxygen-dependent hydroxylation and degradation [17].

2.2. Regulation of HIF

In normoxia, HIF-α protein subunits have a short half-life (< 5 min) since they are continually transcribed, translated and rapidly degraded [7]. This degradation is mediated by the post-translational

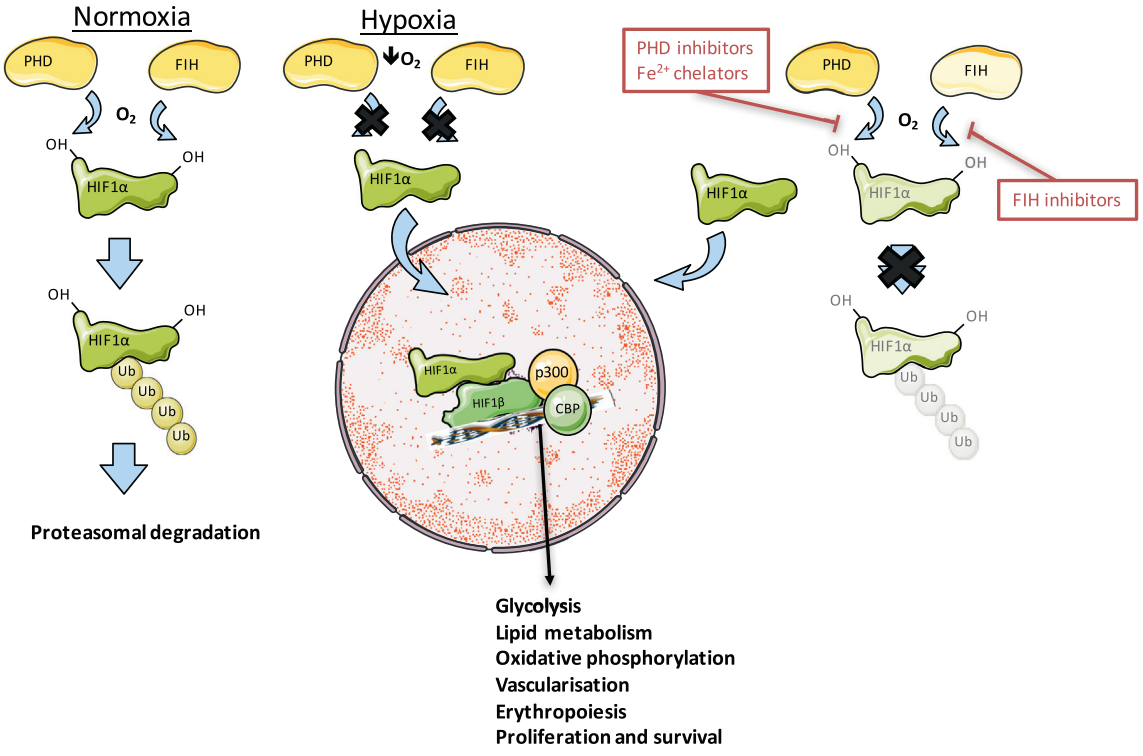

Fig. 1. The regulation of HIF-1α. Under normoxic conditions, HIF-1α is constantly synthesized, hydroxylated and degraded. This hydroxylation occurs through the action of Prolyl Hydroxylase Domain (PHD) and Factor Inhibiting HIF (FIH) enzymes, which are oxygen dependent. Once hydroxylated, HIF-1α is then poly-ubiquitinated and undergoes subsequent proteasomal degradation. In hypoxia, the PHDs and FIH are inhibited by lack of their substrate oxygen so cannot hydroxylate HIF1-α, which is therefore stabilized and able to translocate to the nucleus, where it binds HIF1-β. This complex recruits co-activators of transcription p300 and CBP, which allows the initiation of transcription of target genes. Compounds developed as hypoxia-mimetics target either the PHDs or FIH, preventing hydroxylation of HIF-1α and allowing for stabilisation and transcription of target genes under normoxic conditions.

hydroxylation of highly-conserved proline residues (Pro402 and Pro564 in HIF-1α, Pro405 and Pro531 in HIF-2α) within their NODD and CODD domains, by specific prolyl hydroxylase domain enzymes (PHDs) [18]. This hydroxylation forms a binding site for the von Hippel-Lindau tumor suppressor protein (pVHL), which forms the substrate recognition component of the multiprotein E3 ubiquitin ligase complex (alongside the transcription factors Elongin B and Elongin C, cullin-2 and ring-box 1) [19]. pVHL catalyses the polyubiquitination of specific lysine residues in HIF-1α, marking it for proteasomal degradation [20]. Since the regulatory hydroxylases are oxygen-dependent, they are inhibited under hypoxic conditions, and hence pVHL does not bind to the HIF-α subunit in hypoxia to target it for degradation. This results in stabilisation and accumulation of HIF-α subunits, which can translocate to the nucleus to dimerise with HIF-β. Other signalling pathways have since been shown to interact with HIF, however the PHD-VHL-HIF axis is now widely-regarded as the primary control of the cellular hypoxic response [21–24].

The prolyl hydroxylase domain enzymes (also known as EGLN1-3) responsible for HIF prolyl hydroxylation exist as three different isoforms, PHD1-3. These three isoforms are near ubiquitously expressed, but with large differences in expression levels between tissues [25]. PHD1 is exclusively nuclear, with PHD2 predominantly located in the cytoplasm, while PHD3 can be found in both [11]. A fourth enzyme (P4H-TM), found in the endoplasmic reticulum, permits in vitro HIF-α hydroxylation [23]. On a tissue level, PHD2 and 3 are more highly expressed in the heart relative to other tissues [26]. The different PHD isoforms manifest different affinities and specificities for each HIF isoform. PHD2 and PHD3 mRNA levels are increased by hypoxia [26,27], and are transcriptional targets of HIF-1, forming a feedback loop in hypoxia [28]. The expression of multiple isoforms of PHDs in the same tissue has raised questions regarding differences in regulation and targets [29]. It has been postulated that PHD2 likely acts as the primary rate-limiting HIF prolyl hydroxylase in normoxia, while the other PHD isoforms affect HIF activity during chronic hypoxia. Interestingly, PHD2 silencing has been shown to impact to a greater extent on HIF-1α, whereas PHD3 impacts on HIF2α [29].

The prolyl hydroxylases form part of the non-heme Fe(II) and 2-oxoglutarate (2OG)-dependent dioxygenase superfamily, with a conserved two-histidine-one-carboxylate Fe(II) coordination motif, two positions for binding 2-OG and one for molecular oxygen. Each oxygen bound is split in two; one atom is used to catalyse the oxidative decarboxylation of 2-oxoglutarate (α-ketoglutarate) into succinate and CO_2 and the other is incorporated into the hydroxylated amino acid residue of HIF-α [30] (with a highly-reactive ferryl intermediate formed during the process). The unusually slow reaction of the hydroxylase with oxygen, as demonstrated kinetically, is thought to be a key factor in facilitating the enzyme's role as a hypoxia sensor [31].

HIF is also regulated by a second round of post-translational modification, which affects its transcriptional activity rather than its stability. Another independent Fe(II)- and 2-oxoglutarate dependent dioxygenase, FIH, catalyses the hydroxylation of conserved asparagine residues in the HIF-α CAD (N803 in HIF-1α, N847 in HIF-2α) under normoxic conditions. This asparagine hydroxylation of HIFα likely results in a direct steric clash with the coactivator protein p300 necessary for transcriptional expression of most HIF target genes. Like the PHDs, FIH is also inhibited by hypoxia, but to a lesser extent, so it may perform a 'fine-tuning' role, to inhibit HIF-α proteins not targeted by PHDs in moderate hypoxia [32,33]. Furthermore, FIH exerts a stronger inhibitory effect on HIF-1α than it does on HIF-2α, which suggests that some HIF target genes require a greater level of hypoxia than others to be expressed [34].

2.3. Regulatory control of HIF hydroxylases

Studies suggest that the prolyl hydroxylases and FIH may also be controlled at several levels independently of oxygen tension, allowing greater flexibility within the hypoxic response. Nitric oxide donors may be used to competitively inhibit the HIF hydroxylases in vitro and increase their transcriptional activity [35], however, recent studies have produced conflicting results [36,37]. As the HIF hydroxylases are Fe-dependent dioxygenases, changes in Fe levels have been shown to modulate HIF-α stabilisation [38]. Of interest, levels of Krebs cycle intermediates, particularly succinate (the product of hydroxylase reaction) and fumarate, exert regulatory effects on the HIF hydroxylase enzymes. Mutations in Krebs cycle enzymes in cancer provide the strongest evidence to date: genetic defects in succinate dehydrogenase elevate succinate levels and upregulate HIF-α in normoxia, associated with oncogenesis [39]. Similarly, mutations in fumarate hydratase (fumarase) stabilise HIF-1α, creating a state of pseudohypoxia [40,41]. We have recently shown this Krebs-cycle intermediate-dependent phenomenon in the heart, whereby changes in myocardial succinate in hypoxia regulate cardiac HIF-1α stabilisation [42]. The proposed mechanism linking abnormal Krebs cycle metabolism to HIF hydroxylase inhibition is via feedback inhibition of the regulatory enzymes.

2.4. HIF target genes

HIFα/β complex binds to hypoxia response elements (HREs) at target gene loci, regulating expression of a network of genes involved in the hypoxic response on both a cellular and systemic level. The HREs share one or more common binding sites for HIF, known as HIF-binding sites (HBSs) [43], as well as neighbouring DNA binding sites for additional transcription factors, which must also be bound to allow gene transactivation. The mandatory core consensus sequence within the HBS of the HRE is NCGTG (where N is A or G). Epigenetic mechanisms, such as CpG methylation [44] and oxidative DNA damage caused by reactive oxygen species (ROS) also affect HIF binding to the HRE [45].

HIF has been shown to be a master transcription factor, regulating many hundreds of target genes in response to hypoxia [46]. Broadly, HIF targets are aimed at adapting the cell to this new lower oxygen environment, downregulating processes that consume oxygen, upregulating anaerobic processes, as well as aiming to restore normal oxygen delivery.

The hypoxia-responsive genes may respond exclusively to HIF-1, to HIF-2, or respond to both. HIF-1 preferentially promotes the expression of genes encoding enzymes involved in the glycolytic pathway such as phosphoglycerate kinase (PGK) and lactate dehydrogenase A (LDHA). HIF-2, on the other hand, preferentially targets genes that are involved in invasion, for example Oct-4 and TGF-α [47]. However, HIF-1 and -2 also share some overlapping target genes, including those that encode vascular endothelial growth factor A (VEGFA) and glucose transporter 1 (GLUT1) [47]. Moreover, the two isoforms demonstrate an ability to compensate for the absence of the other by performing each other's isoform-specific functions [48,49]. Given the tissue-specific isoform expression patterns of both the HIF transcription factors and their regulatory HIF hydroxylases, coupled with different degrees of hypoxia between various tissues, differences in gene expression have been identified between tissues and between hypoxia-associated diseases.

In addition to upregulating target genes, HIF activation has been shown to decrease expression of hundreds of genes. Examples of this include genes that drive oxygen consumption and oxidative metabolism [50,51]. In a recent study, there was no significant association between the presence of an HBS and downregulation of the gene by hypoxia, demonstrating that the downregulation of target proteins is via indirect mechanisms [46].

Metabolism is a major target for adaptation following HIF activation, as oxygen is the terminal electron acceptor in oxidative phosphorylation. During hypoxia, cellular metabolism is shifted away from the oxygen-dependent process of oxidative phosphorylation, to favour the oxygen-sparing glycolytic pathway [52]. This allows cells to generate ATP in conditions of lower oxygen tensions. HIF regulates the expression of multiple genes that promote oxygen delivery through

increased erythropoiesis (such as erythropoietin) and angiogenesis (such as VEGF), to try to restore the cell back to its previous normoxic environment [53,54].

3. Metabolism in the healthy heart

In order to maintain ceaseless contractile activity, the heart requires a constant supply of energy, in the form of ATP. The human heart synthesises approximately 6 kg of ATP daily [55], generated predominantly via mitochondrial oxidative phosphorylation [56]. The heart is a metabolic omnivore, capable of metabolising various substrates including fatty acids, glucose, pyruvate and lactate, to satisfy its energy demands [57,58]. Cardiac fuel substrate selection is dynamic and may be altered according to the prevailing physiological or pathophysiological conditions. This metabolic flexibility allows the heart to maintain ATP production under varying physiological conditions, and is primarily regulated by substrate concentration, hormone levels, coronary blood flow, oxygen supply and cardiac workload [59,60]. Fatty acids yield more ATP per carbon atom than glucose, pyruvate or lactate, as a result, the healthy heart preferentially uses fatty acid as a fuel source under steady state conditions [55]. In the healthy heart, fatty acid metabolism accounts for approximately 60–70% of energy metabolism in the fasted state [61]. However, in response to feeding, insulin-stimulated glucose metabolism becomes the predominant fuel. This metabolic flexibility between fuels allows the heart to match substrate use to the physiological conditions.

3.1. Fatty acid uptake and metabolism

Long chain fatty acids (LCFA) are hydrophobic and insoluble in aqueous environments, so they are transported to the heart bound to albumin as non-esterified fatty acids (NEFAs) or esterified to triacylglycerol and incorporated into lipoproteins, such as chylomicrons [62,63]. The uptake of NEFAs into myocardial cells can occur passively across cell membranes, but primarily uptake is mediated by transporter mechanisms involving fatty acid translocase (FAT/CD36), plasma membrane fatty acid binding protein (FABPpm) and fatty acid transport proteins (FATP1 and 6, Fig. 2). Cytosolic fatty acids are esterified by acyl-CoA synthetase on the mitochondrial membrane, forming long chain fatty acyl coenzyme A. Carnitine palmitoyltransferase-1 (CPT1) in the mitochondrial intermembrane space substitutes the CoA group of the long chain acyl-CoA with carnitine, and carnitine acyltranslocase (CAT) shuttles fatty acyl-carnitine across the inner mitochondrial membrane in exchange for free carnitine. In the inner membrane, CPTII converts the fatty acyl-carnitine back to long chain fatty acyl-CoA in the mitochondrial matrix. CPT1 serves as a key regulatory step in mitochondrial fatty acid oxidation, due to its allosteric inhibition by malonyl CoA (derived from acetyl CoA carboxylase in the fatty acid synthetic pathway). The long chain fatty acyl-CoA undergoes β-oxidation in the mitochondrial matrix, resulting in the production of acetyl-CoA and reduced cofactors NADH + H^+ and $FADH_2$, which enter the electron transport chain to produce ATP [56,64].

3.2. Glucose uptake and metabolism

Glucose is transported into the cytosol by glucose transporters, including GLUT1 and GLUT4 (Fig. 3). GLUT1 is responsible for basal glucose uptake and constitutively present in the sarcolemma [65], whereas GLUT4 cycles between the sarcolemma and intracellular vesicles in response to insulin and AMP-activated protein kinase (AMPK) activation, and therefore serves as the predominant regulator of glucose uptake [66]. When glucose is taken up in cardiomyocytes, it is rapidly phosphorylated by the enzyme hexokinase to glucose-6-phosphate, which subsequently can enter many metabolic pathways including glycolysis, glycogenesis, the pentose phosphate and hexosamine biosynthetic pathways. Glycolysis represents a major pathway for glucose utilization in the heart. Glycolysis generates pyruvate, NADH + H^+ and a small quantity of ATP via substrate level phosphorylation, through a series of cytosolic reactions that can operate under oxygen-independent conditions. PFK is a key regulatory enzyme in the glycolytic pathway, allosterically regulated by the cellular energy status and metabolite concentrations [67,68]. The end product of glycolysis, pyruvate, can either enter the mitochondria for oxidation or be reduced to lactate in the cytosol. Under aerobic conditions, pyruvate is decarboxylated to acetyl-CoA within the mitochondria, prior to entry into the Krebs cycle for glucose oxidation (Fig. 3). Mitochondrial pyruvate dehydrogenase (PDH) catalyses this oxidative decarboxylation of pyruvate, and is the key regulated enzyme complex in glucose metabolism [69]. PDH is phosphorylated and inhibited by pyruvate dehydrogenase kinase (PDK) enzymes 1–4, which are activated by the NADH + H^+ and acetyl-CoA. In contrast, PDH is dephosphorylated and activated by pyruvate dehydrogenase phosphatase (PDP), which is activated by insulin and calcium [70]. Under anaerobic conditions, pyruvate is reduced to lactate for export by the action of lactate dehydrogenase, utilizing glycolytically-derived NADH + H^+.

3.3. Myocardial energetics

Glucose and fatty acid metabolic pathways converge at acetyl-CoA, which enters into the mitochondrial Krebs cycle, generating mitochondrial NADH + H^+ and $FADH_2$. These reducing equivalents donate electrons that are shuttled along the electron transport chain (ETC) in the inner mitochondrial membrane between complexes I to IV. The electrons are ultimately donated to oxygen at complex IV, producing H_2O. Concomitantly, complex I, III and IV of the ETC utilize the energy released in these redox reactions to pump protons into the mitochondrial intermembrane space, generating an electrochemical gradient across the inner membrane. This gradient is the driving force for the phosphorylation of ADP to ATP by F_1F_o ATP synthase [71,72].

4. Metabolism in the hypoxic heart

Given that the majority of ATP required by the heart is generated by mitochondrial oxidative phosphorylation, oxygen plays a critical role in cardiac energy metabolism. When oxygen availability is limited, the heart is metabolically remodelled, and shifts away from oxygen-demanding substrates to more oxygen-efficient fuels. Although long chain fatty acids are more energy dense than glucose (ideal for making large amounts of ATP), they also need large amounts of oxygen to generate that ATP. In contrast, complete metabolism of glucose yields 11% more ATP per oxygen atom consumed than fatty acids [55]. Collectively, many studies demonstrated that chronic hypoxia triggers regulatory pathways that mediate cardiac metabolic remodelling, particularly at the transcriptional level, to sustain energy production and contractile function under oxygen-limited conditions [73,74]. This includes adaptation to hypoxia in the context of a myocardial infarction (MI), but also chronic disruptions of oxygen delivery, such as chronic intermittent hypoxia, sleep apnoea, or anaemia.

4.1. Fatty acid uptake and metabolism

Data on myocardial fuel substrate preferences in humans exposed to chronic hypoxia is limited, however low-landers acclimatised to high-altitude displayed decreased reliance on fatty acid metabolism [75,76]. The most frequently used experimental model in animal studies of hypoxia are simulated in normobaric or hypobaric chambers, which have been employed most commonly in a simulated altitude of approximately 5500 m (11% oxygen). Molecular findings in response to chronic hypoxia indicated that the putative regulatory steps orchestrating substrate switching away from fatty acid metabolism may include a) attenuated sarcolemmal fatty acid uptake, b) reduced mitochondrial fatty acid uptake and oxidation, and c) increased malonyl-

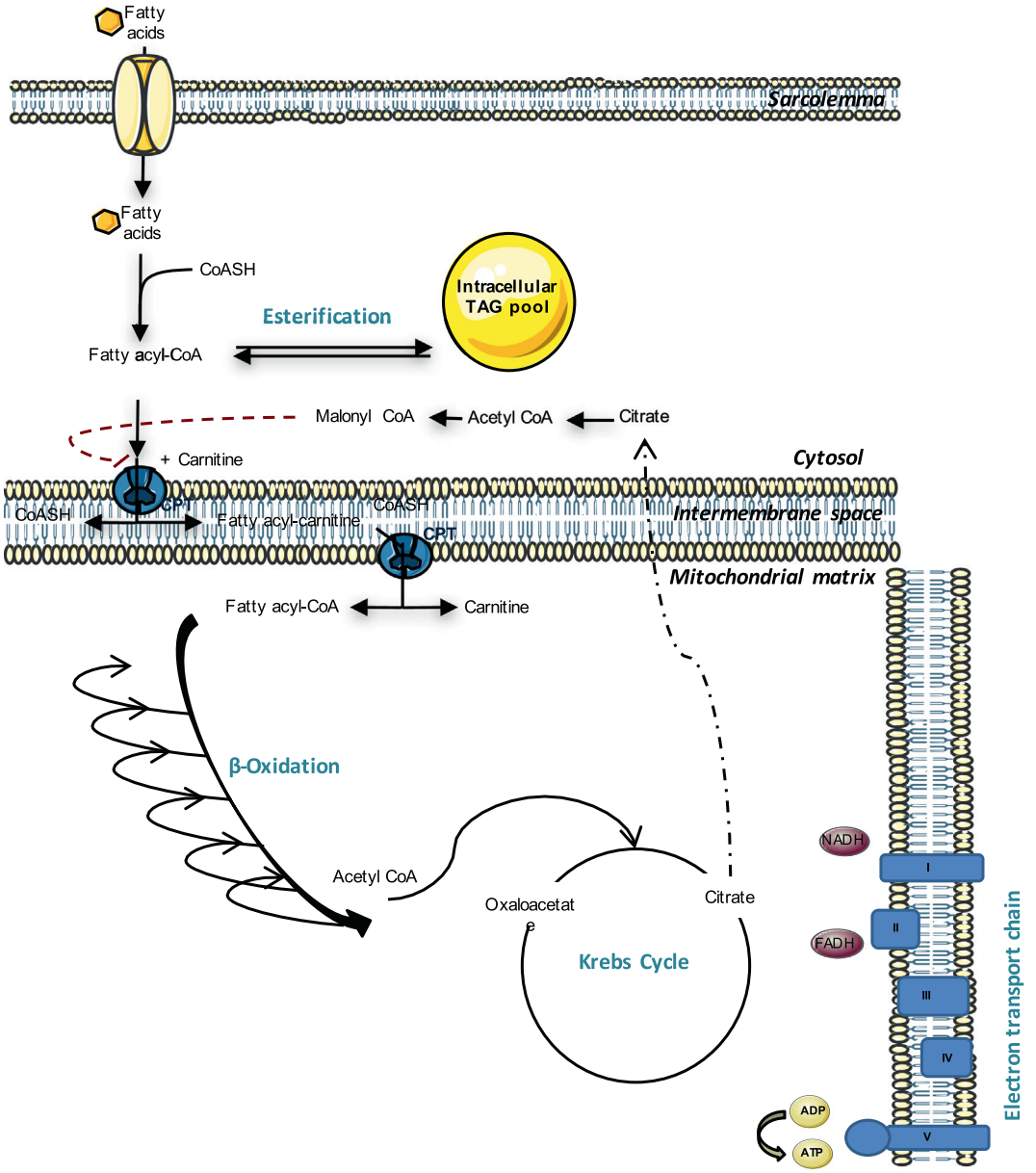

Fig. 2. Myocardial fatty acid utilization. Non-esterified fatty acids (NEFA) enter the cardiomyocyte either through passive mechanisms or through transporters such as FAT/CD36, FABPm and FATP. NEFA can then undergo esterification to fatty acyl-CoA and enter the cellular triacylglyceride pool or enter the mitochondria. Transportation into the mitochondria occurs through CPT1, using carnitine. This can be allosterically inhibited by malonyl-CoA, as a feedback inhibition mechanism. Once in the mitochondrial matrix, fatty acyl-CoA can undergo β –oxidation, producing acetyl-CoA to be used in the Krebs cycle, and reduced cofactors NADH + H^+ and $FADH_2$, which enter the electron transport chain to produce ATP [56,64].

CoA levels, allosterically inhibiting CPT1 activity.

Chronically hypoxic rat hearts have decreased protein levels of FATP1 [77] which could potentially decrease the capacity for fatty acid uptake across the sarcolemma. Three weeks exposure to 11% oxygen, markedly decreased β-oxidation rates in both mouse [73] and rat [77] hearts. Cardiac mitochondria have decreased ADP-stimulated state 3 respiration with fatty acid substrates following chronic hypoxia [78]. This is in agreement with decreased enzyme activity for fatty acid oxidation enzymes: CPT1, β-hydroxy-acyl-CoA dehydrogenase [79,80] and medium-chain acyl coenzyme A dehydrogenase (MCAD) [81].

Hypoxia-induced cardiac fatty acid metabolic remodelling is tightly regulated by the nuclear transcription factor peroxisome proliferator-activated receptor alpha (PPARα). PPARα has been shown to negatively correlate with blood haemoglobin content in high altitude tolerant humans [82], and hypoxic animal models [81,83]. PPARα transcriptionally regulates numerous genes that control cardiac fatty acid mitochondrial import and oxidation, including FATP1, MCAD and CPT1 [84,85], which were shown to be down regulated in parallel with decreased PPARα [73,81] expression following 3–5 weeks of chronic normobaric hypoxia (Fig. 4). Decreased PPARα expression and CPTI have also been reported in other models of systemic hypoxia, induced by chemical induction using cobalt chloride treatment and isovolaemic dilution [86]. In rat cardiomyocytes, HIF-1 was involved in hypoxia-induced suppression of fatty acid metabolism by decreasing the DNA binding activity of PPARα and its coactivator retinoid X receptor (RXR) [87]. PHD1-deficient mice, a model with chronic HIF upregulation, have increased PPARα expression in skeletal muscle [88]. In HL-1 cardiomyocytes, hypoxia or prolyl hydroxylase inhibition increased both HIF-1α and HIF-2α, and decreased PPARα and its target genes [73]. Metabolic remodelling mediated by PPARα downregulation was shown to be essential to maintain ATP production and cardiac function during chronic hypoxia; and the disruption of metabolic adaptation via

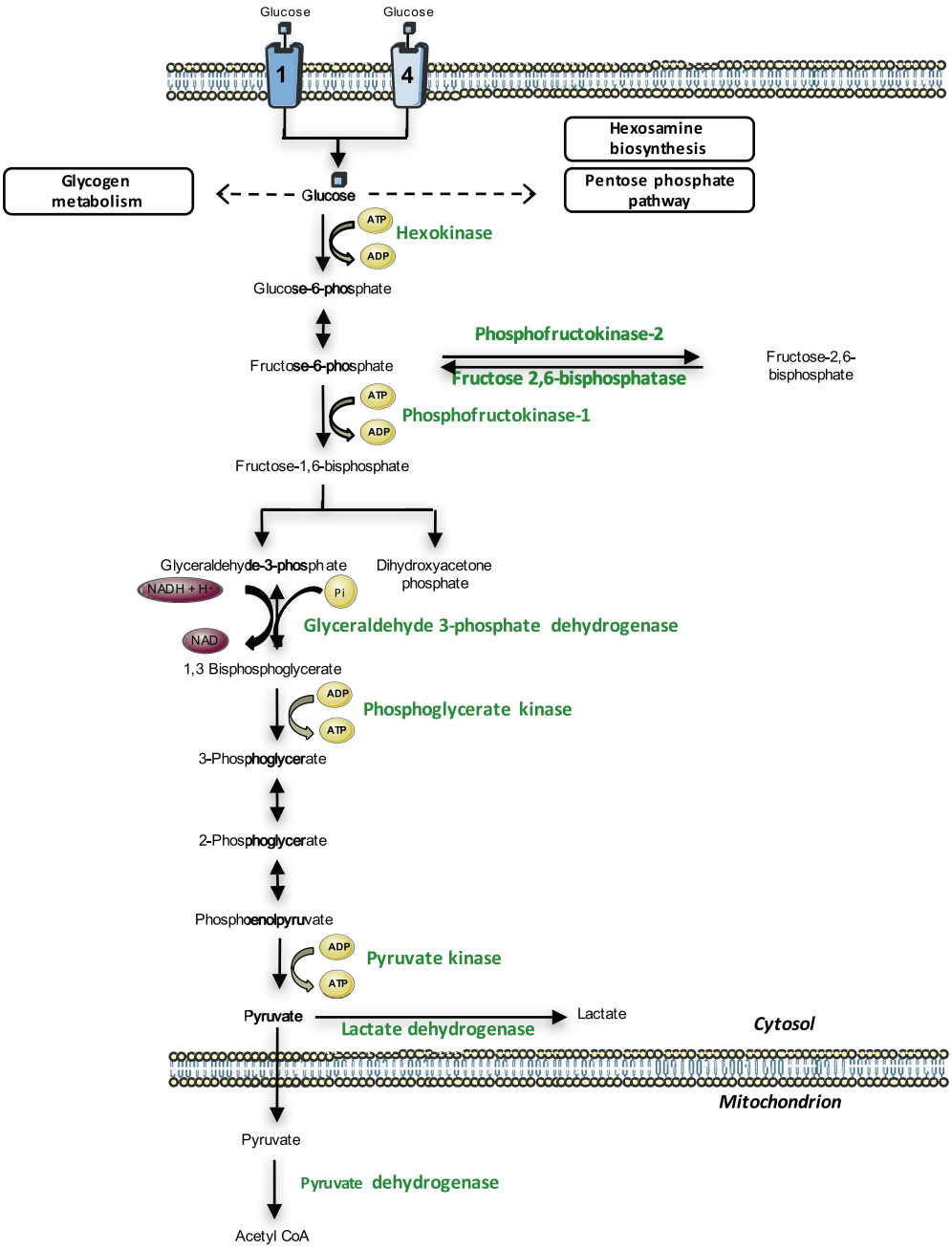

Fig. 3. Myocardial glucose utilization. Glucose enters the cardiomyocyte through glucose transporters GLUT1 and 4. It is then rapidly phosphorylated by the enzyme hexokinase to glucose-6-phosphate, which subsequently enters many metabolic pathways including glycolysis, glycogenesis, the pentose phosphate and hexosamine biosynthetic pathways, though glycolysis is the main pathway for glucose use in the heart. Glycolysis produces a small quantity of ATP anaerobically, as well as generating pyruvate, which can enter the mitochondria for oxidation. Mitochondrial pyruvate dehydrogenase (PDH) catalyses the oxidative decarboxylation of pyruvate, and is the key regulated enzyme complex in glucose metabolism.

manipulating PPARα, achieved by high fat feeding and genetic ablation of PPARα, impaired hypoxic adaptation and resulted in cardiac dysfunction [73].

4.2. Glucose uptake and metabolism

In human, both high-altitude adapted natives and low-landers exposed to altitude simulations displayed enhanced cardiac glucose uptake [89,90]. In rodents, exposure to chronic hypoxia increased glucose uptake [91] and the activities of cardiac glycolytic enzymes including hexokinase [79,92], pyruvate kinase, PFK (both PFK-1 and the inducible form of the enzyme, PFK-2) [79,93] and LDH [79,94], parallel to augmented glycolytic flux [73,77]. Many of these glycolytic targets have been demonstrated in non-cardiac tissue to be driven directly by the HIF system [95]. Findings on glucose transporters have been inconclusive in the heart; mRNA expression of GLUT1 was not altered after 4 weeks of chronic hypoxia [83] but at the protein level GLUT1 has been reported to increase after 2 weeks, while GLUT 4 protein levels remain unchanged [73,96,97].

Many studies have indicated that in non-cardiac tissues glycolysis is uncoupled from the Krebs cycle in hypoxia, via transcriptional activation of PDK1 driven by HIF [98,99]. PDK1 phosphorylates and inhibits PDH, directing the fate of pyruvate towards lactate production instead of oxidation. This hypoxia-induced metabolic switch shunts glucose derivatives away from the mitochondria. In heart this mechanism does not appear as clear-cut. Hypoxic HL1-cardiomyocytes have increased PDK1 protein levels [100], however, PDH complex activity was not changed in the heart in response to in vivo chronic hypoxia [101]. Cardiac PDK4 is consistently decreased in many hypoxia studies [83,86], as PDK4 is a downstream target of PPARα.

Certainly, lactate efflux from the heart is increased in response to chronic hypoxia [73,97] (Fig. 4). This is supported by multiple studies which have shown higher expression of the plasma membrane lactate transporter, monocarboxylate transporter MCT4, as well as cardiac

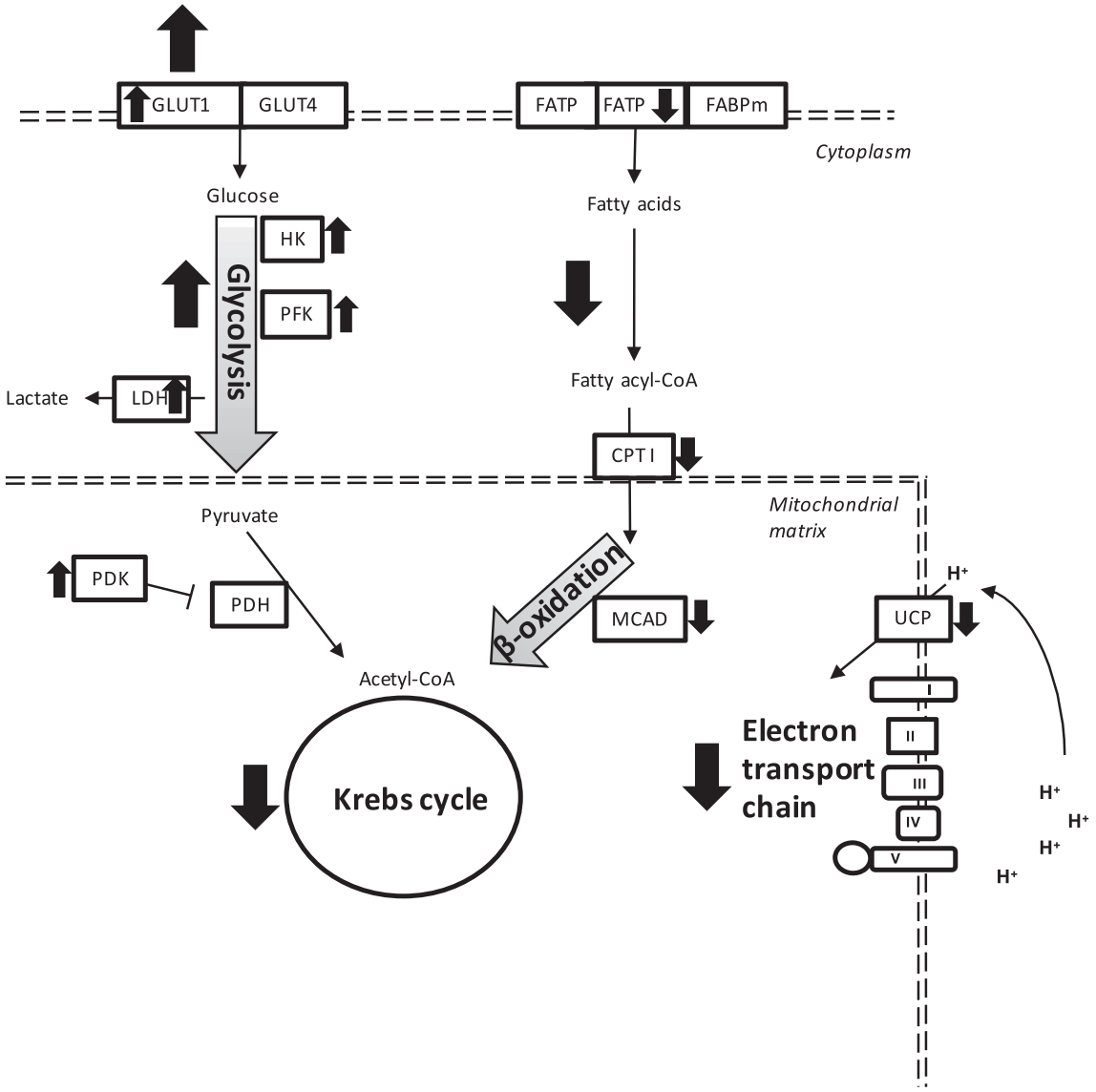

Fig. 4. Cellular metabolic effects of HIF-1. HIF-1 activation leads to the upregulation of a number of targets genes, many of which are involved in cellular metabolism. Key metabolic targets include glucose transporter 1 (GLUT1), hexokinase (HK), phosphofructokinase (PFK), lactate dehydrogenase (LDH), and pyruvate dehydrogenase kinase (PDK), all of which lead to an increase in glucose utilization, upregulating the glycolytic pathway. In addition, via activation of PPARα, HIF-1 activation results in a decrease of proteins involved in fatty acid uptake and oxidation, namely fatty acid transport protein (FATP), carnitine palmitoyltransferase I (CPTI), medium chain acyl-CoA dehydrogenase (MCAD), and uncoupling proteins (UCP). Together, this results in an overall decrease in oxygen-demanding fatty acid oxidation. Consequently, the activity of the electron transport chain (ETC) is also downregulated by HIF-1 activation.

lactate dehydrogenase expression in response in vitro and in vivo hypoxia [102,103]. Increased net lactate efflux is proportional to the increase in glycolytic flux with hypoxia, thus the ratio of lactate production to glucose uptake was still maintained, indicative of unchanged pyruvate oxidation rate [73,77].

4.3. Mitochondrial respiration and myocardial energetics

Following chronic in vivo hypoxia, decreased isolated mitochondrial respiration with fatty acid and pyruvate substrates has been consistently reported [78,104,105]. We have shown previously that in response to hypoxia not all mitochondria within the heart adapt in the same way, and that glutamate respiration is decreased in subsarcolemmal mitochondria (SSM) but not in interfibrillar mitochondria (IFM) [78]. This is because SSM adapt to hypoxia by modulating their electron transport chain activity, whereas IFM modulate their Krebs cycle, upstream of where glutamate derivatives enter the Krebs cycle. Hypoxia decreases enzymatic activities of electron transport chain complexes I, II and IV in SSM, and aconitase in IFM [106]. The decrease of complex I might serve to decrease ROS generation but potentially at the cost of decreased ATP synthesis [104]. The mitochondrial inner membrane uncoupling protein UCP3 was decreased by hypoxia [73,77,105], another PPARα target gene [56] (Fig. 4). The reduction in UCP3 with hypoxia may serve to increase mitochondrial efficiency of oxidative phosphorylation, either directly or via its involvement in fatty acid oxidation. Indeed, mitochondrial changes may underlie the observed depression in cardiac energetics following hypoxia.

In non-cardiac tissue, HIF maintains respiratory efficiency by targeting other mitochondrial proteins, by inducing a complex IV cytochrome *c* oxidase (COX) 4-1 to COX4-2 subunit switch [50], by inhibiting expression of ISCU, an iron-sulfur cluster assembly factor that is required for activity of aconitase and electron transport complex I [109], and by inducing BNIP3, which triggers mitophagy following prolonged hypoxia [110].

In humans, changes in myocardial energetics have been reported in response to chronic hypoxia. Acclimatisation to a normobaric hypoxia chamber decreased cardiac phosphocreatine to ATP (PCr/ATP) ratios by 15% [111]. Similarly, 11 days at altitude of 5300 m at Everest Base Camp decreased PCr/ATP ratio by 18% [112]. In Sherpas indigenous to living at altitude, cardiac PCr/ATP ratios were significantly lower than native lowlanders, even after a month at low altitude [113]. In animal models, decreased cardiac PCr/ATP ratios in chronically hypoxic mouse hearts [73,114,115] was also observed as a result of decreased phosphocreatine levels, with a subsequent increase in cytosolic ADP. Therefore, metabolic remodelling in hypoxia and activation of the HIF system is a fundamental adaptation of the heart to ensure continuous ATP production, by guiding substrate switching towards anaerobic glycolysis. The need for this metabolic flexibility is particularly important in the context of the current epidemic of cardiovascular disease.

5. Cardiovascular diseases and metabolism

Cardiovascular disease is the leading cause of death worldwide, and accounts for approximately 17.3 million deaths per year [116]. Myocardial infarction occurs when a thrombus causes occlusion of the coronary circulation, resulting in ischaemia in the area no longer perfused by the affected artery [117]. The ischaemic tissue is no longer receiving oxygen or nutrients, and is not being cleared of waste products. During the period of ischaemia, the lack of supply to the affected tissue causes ischaemic injury, which includes failure of energy metabolism, dysregulated calcium homeostasis, generation of free radicals and mitochondrial dysfunction [118]. The survival of the myocardium is dependent on several factors, namely the severity of the occlusion, the duration of ischaemia, the size/location of the area affected by the occlusion, and how the cardiomyocyte adapts to the ischemic insult. It is already well known that promoting new blood vessel growth can improve survival of the myocardium [120]. This highlights the key role for collateral blood vessel formation and ischaemia-induced angiogenesis in response to myocardial infarction.

Taking into account the heart's high metabolic demand, it comes as no surprise that cardiovascular diseases involve marked changes in myocardial metabolism. Many studies have assessed the metabolic changes that take place in the heart during the progression to heart failure, assessing animal models of cardiac hypertrophy and failure, as well as patients with heart failure. Though the results have not always been consistent, the consensus is that fatty acid use is decreased in almost all models of hypertrophy and heart failure, and this is most often accompanied with an increase in glycolysis and glucose oxidation. Patients with idiopathic dilated cardiomyopathy, who exhibited systolic dysfunction, showed decreases in markers of fatty acid utilization and evidence for increased glucose use [121,122]. Similarly left ventricular hypertrophy, induced by aortic banding or by myocardial infarction, is associated with decreased fatty acid oxidation and increased glucose use [123,124]. It is important to note that the progression to heart failure is a slow, complex process and, for this reason, the metabolic changes observed in cardiac hypertrophy and heart failure will vary depending on aetiology, progression, and severity and stage of disease. In pathologic hypertrophy, hypoxia induces HIF-1α expression [125], which plays an important role in reprogramming myocardial metabolism. The initial metabolic reprogramming, which involves an adaptive increase in glucose uptake and glycolysis, is likely to be due to the hypoxia that results from the mismatch between oxygen supply and demand [126]. The transition to heart failure involves changes in metabolic targets involved in glycolysis and PDH flux in patients at the later stages of hypertrophy [86]. Additionally, the transition to heart failure has also been associated with changes in fatty acid oxidation markers, namely PPARα targets [127].

Diabetes is a growing concern worldwide and a systemic metabolic disease, which has been recognised as an independent risk factor for the development of heart failure, with a significant decrease in survival rate following MI compared to non-diabetic patients [128–130]. Diabetic cardiomyopathy (DCM) is a specific pathology associated with diabetes mellitus, which is characterised by structural and functional cardiac changes, including fibrosis, ventricular dilation and diastolic dysfunction [131]. In diabetes mellitus, energy metabolism is greatly reshaped, due to a dysregulation of glucose metabolism and fatty acid metabolism. From a metabolic perspective, type 2 diabetes is characterised by increased cardiac fatty acid oxidation and an inability to use glucose due to insulin resistance [132], and as a consequence, cardiac metabolic flexibility is impaired [59]. It is likely that early in obesity, prior to type 2 diabetes, the changes in substrate use relate to substrate availability, namely the increase in circulating fatty acids and triglycerides. This, however, leads to an adaptation of the cardiac metabolic machinery in response to changing conditions, leading to decreased glucose use as a result of increased fatty acid use via the Randle cycle [129,133]. The resulting hyperglycaemia and hyperlipidaemia, together with insulin resistance, lead to changes in the expression and translocation of GLUT4 and FAT/CD36, with increased translocation of FAT/CD36 towards the sarcolemma and decreased sarcolemmal GLUT4 [134]. Increased fatty acid uptake is associated with increased fatty acid oxidation, activation of PPARα and its fatty acid metabolism target genes and increased lipid storage. These fatty acid metabolic changes ultimately result in a decreased ability to use glucose, and an associated loss of diastolic function, which has been observed in rodent models of obesity with insulin resistance [135], diabetes [136] and in patients with type 2 diabetes with no concomitant cardiovascular symptoms [137].

6. HIF signalling in cardiovascular diseases

Ischaemia inevitably results in local hypoxia, which elicits a hypoxic response in the affected tissue and neighbouring region. HIF signalling is stimulated in these hypoxic conditions [125]. This allows increased expression of its target genes, which leads to a range of beneficial effects including angiogenesis and the aforementioned metabolic adaptations to hypoxia. This response is key in supporting the tissue to cope with the ischaemic insult and alleviate the subsequent deleterious longer-term effects of ischaemia-reperfusion. HIF-1 activity has been shown to increase early following MI, in ventricular biopsies from patients undergoing coronary bypass surgery [138]. Studies using coronary ligation in the rat heart showed increased expression of HIF-1α protein and HIF-1 targets mRNA over the first week following infarction [26]. In addition, Wei et al. found that HIF-1α conditional KO mice quickly developed cardiac hypertrophy and decompensation when subjected to pressure overload, and concluded that HIF-1 plays a crucial role in protecting the myocardium in the development of hypertrophy [139]. In line with this, Cai et al showed that protective effect of intermittent hypoxia was lost in HIF-1α $^{+/-}$ mice [140]. In contrast, work in a transgenic mouse model of constitutively active HIF-1α showed reduced infarct size and improved cardiac function four weeks after coronary ligation [141].

The activation of HIF-1 in endothelial cells during hypoxia or ischaemia is equally crucial to the subsequent recovery of the heart. As well as having metabolic oxygen sparing effects on the endothelium, HIF-1 mediates the angiogenic response to hypoxia by upregulating the expression of angiogenic factors. A genetic model of prolyl hydroxylase inhibition showed that activation of HIF-1 signalling in endothelial cells resulted in improved LV function and survival following ischaemia-reperfusion [142]. Furthermore, a lack of concordance between myocardial growth and endothelial angiogenesis has been shown to contribute to the development of heart failure [143]. Angiogenic HIF-1 targets such as VEGF are responsible for the fast growth of collateral vessels and have been implicated in cell survival following myocardial infarction [138,144]. Polymorphisms in the HIF-1α locus have been associated with reduced collateral vessel formation, affecting the progression of ischaemic disease [145]. Furthermore, expression of target

Table 1
Classes and examples of HIF activators.

Class	Compound	Current use
2-OG mimetics	Dimethyloxalylglycine	Tool compound – PHD inhibitor
	N-oxalyl-*d*-phenylalanine	Tool compound – FIH inhibitor
Iron chelators	Desferrioxamine	Phase II clinical trials for use in brain haemorrhage
	Hydralazine	Licensed as an antihypertensive, recently found to also have a HIF mechanism of action
	AKB-4924	Phase I clinical trials for the treatment of inflammatory bowel diseases
PHD active-site blockers	GSK360A	Pre-clinical studies showed improved left-ventricular function and remodelling in rat model of established heart failure.
	Daprodustat (GSK1278863)	Phase III clinical trials for use in anaemia associated with chronic kidney disease. Also in Phase II for the treatment of perioperative ischaemia and diabetic foot ulcers.
	Roxadustat (FG-4592)	NDA filed in China for treatment in anaemia associated with chronic kidney disease. Also in Phase III clinical trials for use in anaemia associated with chronic kidney disease
	Molidustat (BAY85-3934)	Phase II clinical trials for use in anaemia associated with chronic kidney disease
	Vadadustat (AKB-6548)	Phase III clinical trials for use in anaemia associated with chronic kidney disease
	JTZ-951	Phase III clinical trials for use in anaemia associated with chronic kidney disease

genes VEGF and HO-1 have been shown to mediate the all-important improvement in cell survival following ischaemia-reperfusion [120,141,146]. Though the initial response mediated by hypoxic signalling protects the myocardium by improving oxygen delivery and preventing oxygen waste, it remains unclear whether the hypoxia response is beneficial in the longer term, as it has become apparent that angiogenic stimuli induce hypertrophy [147]. It is clear that HIF-1 signalling is increased in the initial stages of hypertrophy, but it may not be the case in prolonged hypoxia, as shown in models of pressure overload [146]. This mismatch between persistent hypoxia and decreased HIF-1 signalling could be at the core of the damage and dysfunction observed in the failing heart [147].

Although the role and effects of HIF-1 have been extensively studied over the past two decades, as it was the first identified of this family of transcription factors, the complimentary and contrasting roles of HIF-2 and HIF-3 remain relatively poorly understood to date. For instance, HIF-2 has been shown to have no effect on glycolysis, unlike HIF-1 [9]. Generally, HIF-2 has been found to have effects particularly on longer term adaptation to hypoxia, suggesting a sort of takeover from HIF-1 to HIF-2 with prolonged periods of hypoxia, which appears to be mostly focused on mitochondrial homeostasis [148]. Studies in KO mice suggest HIF-2 may also be important in affecting cell survival during I/R through the regulation of cellular antioxidant capacity [149]. HIF-3 poses a more complex problem to investigate, given that there are alternatively spliced variants of the HIF-3α subunit, adding up to at least seven isoforms [150]. Very little is known about the effects of HIF-3 signalling in the heart. Importantly, HIF-3 has a role in attenuating the activity of HIF-1α, also acting in the transition from HIF-1 to HIF-2 to HIF-3 in sustained [151] hypoxia.

There is a growing body of evidence for impaired HIF-1 activation in diabetes, though there is still some controversy. Specimens of human heart tissue in angina and biopsies from coronary bypass surgery showed decreased HIF-1α and VEGF levels in type 2 diabetic patients compared to non-diabetics [152]. Work in streptozotocin-induced diabetic rats showed increased infarct size in hyperglycaemic rats, which correlated with decreased levels of HIF-1α [153,154]. A negative correlation has been identified between cardiac HIF-1α levels and glycaemic control in patients with type 2 diabetes and in STZ-induced diabetic rats [154,155]. We have recently demonstrated that the mechanism behind HIF-1α impairment in diabetes is of metabolic origin, driven by increased fatty acids. This elevated dependence on fatty acid metabolism suppresses glycolysis, which results in downregulation of myocardial succinate in hypoxia, resulting in decreased HIF-1α stabilisation [42]. It has also been suggested that HIF-1β impairment could precede the development of type 2 diabetes [156].

Studies in both animal models and patients with type 2 diabetes have shown decreased glucose uptake, glycolytic rates, collateral blood vessel formation, and increased ROS production compared to healthy hearts in response to ischaemia [157–161]. We have shown that in response to in vivo chronic hypoxia, type 2 diabetes decreases the upregulation of glycolysis, limits downregulation of fatty acid oxidation, and prevents mitochondrial adaptation [97]. Together, these studies suggest abnormal HIF-1α activity in the diabetic heart, correlating with impaired functional recovery when stressed [153]. One study showed that HIF-1α overexpression in mice prevented cardiac remodelling in diabetic mice [162]. Gu et al. showed that the HIF-1α Pro582Ser polymorphism could confer protection against diabetic nephropathy, by preventing the negative effect of glucose on HIF-1 signalling, suggesting a beneficial effect of HIF-1 on the microvasculature [163].

7. Therapeutic use of HIF activation

HIF-1 activation has already been proven to have therapeutic potential in the context of myocardial ischaemia, through the use of ischaemic pre-conditioning. When tissue is given short bursts of ischaemia-reperfusion before an ischaemic insult, survival is improved and this has been linked to upregulation of the HIF signalling pathway [164]. In a HIF-1α deletion mouse study, when mice underwent preconditioning, the infarct size was reduced in control mice but this was not observed in mice with the HIF-1α deletion [165]. Furthermore, genetic studies using gene silencing to prevent PHD activity led to attenuated ischaemia-reperfusion injury in the heart [166].

HIF-1α -activating pharmacological compounds fall into one of several classes, all of which have their effect by preventing HIF-1α degradation. Examples of these are listed in Table 1. 2-OG mimetics have been used as tool compounds in pre-clinical studies. Pre-treatment with dimethyloxalylglycine (DMOG) in a rabbit model of myocardial ischaemia-reperfusion resulted in a significant decrease in the resulting infarct size following reperfusion [167]. Similarly, hearts from rats pretreated with DMOG showed improved functional recovery following ischaemia-reperfusion in an ex vivo perfused heart setup [168]. In most studies these compounds were administered before the onset of injury or ischaemia, which could put in question their usefulness in a clinical setting. They have also been criticised for their poor selectivity and risk of off-target effects due to their inhibition of other 2-OG dependent enzymes [38,169]. Iron chelators such as desferrioxamine and hydralazine, have been shown to have HIF-1α stabilising properties through PHD inhibition. Desferrioxamine treatment was shown to improve brainstem blood flow and reduce vasospasm in a rat model of aneurysmal subarachnoid haemorrhage [170].

However, new generation PHD inhibitors have since been developed, several of which are undergoing clinical trials for use in conditions such as anaemia associated with renal disease, inflammatory disease, or brain haemorrhage (Table 1). With regards to potential for cardiovascular disease, chronic PHD inhibition strategies have already shown potential in rodent models of heart failure. Treatment with orally-active PHD inhibitor GSK360A resulted in increased expression of HIF targets, accompanied by improved recovery post MI in rats with

established heart failure, showing long term improvements in remodelling and left ventricular function [171]. Roxadustat (FG-4592) has completed Phase I and Phase II trials for use in anaemia, and showed good tolerability, improved circulating haemoglobin, as well as decreased total cholesterol levels, independent of the use of lipid-lowering agents [172–174]. Another orally-available PHD inhibitor [175], Molidustat (BAY85-3934), has completed Phase II clinical trials for treatment of anaemia associated with chronic kidney disease (DIALOGUE trial, NCT02021370), and Phase III clinical trials are currently recruiting (NCT03351166). Vadadustat (AKB-6548) has also undergone Phase II clinical trials, where it showed enhanced iron mobilisation and haemoglobin in patients with chronic kidney disease [176], and showed no significant changes in blood pressure, VEGF or total cholesterol levels [177]. JTZ-951, a recent clinical PHD inhibitor, has completed Phase II clinical trials (NCT01971164), showing safety and tolerability, with improved haemoglobin levels in patients with end-stage renal disease [178]. Some preliminary work has been done which suggests that HIF-1α activation can also be beneficial in diabetes. The clinical PHD inhibitor, Daprodustat (GSK1278863), has now completed Phase I clinical trials for treatment of diabetic foot ulcer (NCT01831804), as well as perioperative ischaemia, suggesting that repurposing of these compounds for use in cardiovascular disease may not be far.

8. Conclusions

In conclusion, HIF-1 signalling plays a key role in the response to cardiovascular diseases, from a metabolic and angiogenic point of view. This evokes HIF-1α as a potential therapeutic target in these diseases, and the recent advances in the development of PHD inhibitors are hopeful in this regard. However, there is still some concern that systemic treatment with hypoxia-mimetic drugs may lead to undesirable effects in healthy tissue, and it is important to note that HIF effects are tissue-specific. For instance, the Vadadustat trial reported several systemic side effects, as well as three deaths, in the treatment arm [176]. In the Roxadustat trial, five patients withdrew due to worsening side effects [174]. Despite being conserved targets for hypoxic adaptation in a number of organs, the regulation of many targets via HIF-1 activation is achieved via different organ-specific mechanisms. Whether sustained HIF-1α upregulation is beneficial or deleterious for the heart is still under debate, and further investigation is needed to better understand the complex mechanisms at play. It may be that there is a cut-off point after which HIF signalling becomes detrimental to cardiac function [179,180]. Additionally, alternative hypoxic signalling mechanisms other than HIF, such as AMPK activation, may also provide a suitable alternative therapeutic target in cardiovascular diseases. HIF-targeting compounds developed so far have been designed with the kidney as their primary target. The current challenge is therefore the development of tissue-specific and PHD-selective inhibitors, which have so far been slow emerging. And although we have alluded to the potential of repurposing of compounds for use in cardiovascular diseases, this will have to be done on a compound-specific basis, with careful assessment of individual compounds.

Transparency document

The Transparency document associated with this article can be found, in online version.

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