Diabetes impairs endothelial cell metabolic reprogramming and angiogenic responses to hypoxia, which are rescued by reconstituted high-density lipoproteins

Khalia R. Primer
South Australian Health and Medical Research Institute

Joanne T.M. Tan
South Australian Health and Medical Research Institute

Peter J. Psaltis
South Australian Health and Medical Research Institute

Christina Bursill (Christina.Bursill@sahmri.com)
South Australian Health and Medical Research Institute

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Abstract

Aims

Diabetes-impaired angiogenic responses to ischemia contribute to vascular complications such as chronic wounds and a poorer prognosis post-myocardial infarction. Diabetes disrupts mechanisms involved in endothelial cell (EC) adaptation to hypoxia, which is essential for angiogenesis. The pyruvate dehydrogenase kinase 4 (PDK4)/pyruvate dehydrogenase complex (PDC) axis is an oxygen-conserving mechanism that preserves EC function in hypoxia, but its role in diabetes-impaired angiogenesis is unknown. We hypothesised that regulation of the PDK4/PDC axis is impaired by diabetes, important in angiogenesis and rescued by pro-angiogenic reconstituted high-density lipoproteins (rHDL).

Methods

In diabetic mice, PBS and rHDL were topically applied daily to full-thickness wounds. Wound PDK4 and phosphorylated PDC (pPDC) were measured by qPCR, western blotting and ELISA. Laser Doppler imaging measured wound blood flow reperfusion and immunohistochemistry assessed CD31$^+$ neovessels. In vitro, human coronary artery ECs were preincubated with rHDL, exposed to high glucose (25Mm) and hypoxia (1.2% $O_2$). Matrigel tubulogenesis and Boyden chambers assessed angiogenic functions, and the Seahorse Bioanalyser assessed cellular respiration. PDK4 was knocked down by siRNA, and chromatin immunoprecipitation determined transcription factor binding to the PDK4 promoter.

Results

Diabetes impaired the early induction of wound PDK4 and pPDC post-wound ischemia. Topical rHDL rescued this, enhancing PDK4 (68%, $P<0.05$) and pPDC (165%, $P<0.01$) levels. This was associated with increased wound neovascularization (62%, $P<0.05$) and closure (154%, $P<0.0001$). In vitro, PDK4 and pPDC levels were markedly increased in ECs exposed to hypoxia (65%, 70% respectively, $P<0.05$). High glucose prevented further step-wise inductions, and caused an aberrant increase in mitochondrial respiration (19%, $P<0.05$). Importantly, rHDL increased $PDK4$ mRNA levels and pPDC two-fold, returning mitochondrial respiration and EC angiogenic functions to normal glucose levels. PDK4 siRNA knockdown attenuated the proangiogenic effects of rHDL. rHDL appears to mediate these effects by increasing the activity of forkhead box O1 (FOXO1). We also show FOXO1 binds to the PDK4 promoter, which is enhanced by rHDL.

Conclusion

The PDK4/PDC axis response to hypoxia is important for angiogenesis and impaired by diabetes, which can be corrected by rHDL. These findings identify a new mechanism for the proangiogenic effects of
rHDL in diabetes and have implications for its development as an agent that accelerates tissue regeneration.

**Introduction**

Diabetes mellitus (DM) is an increasing global epidemic and poses a significant health and economic challenge [1, 2]. The vascular complications of DM have a major impact on a patient's quality of life and are strongly associated with impaired angiogenic responses to ischemia. Patients with DM exhibit reduced angiogenesis in response to the tissue ischemia caused by athero-occlusions in femoral and coronary arteries [3]. Cutaneous wound healing is also impaired in DM as angiogenic responses to wound ischemia are essential for healing [4]. Patients with DM therefore have a higher risk of lower-limb amputation than their non-diabetic counterparts [2, 4, 5].

Angiogenesis is the process by which ECs respond to tissue ischemia by forming new blood vessels from pre-existing ones. Cellular metabolic adaptation to hypoxia is a key aspect of this response [6], wherein ECs must reduce their oxygen consumption to conserve energy and avoid oxidative stress [7, 8]. To achieve this, ECs reprogram their metabolism to increase anaerobic glycolysis [9] and reduce mitochondrial respiration [10]. An inability to undergo this switch in hypoxia may underpin inadequate neovascularization in DM.

A mechanism which regulates this switch is the mitochondrial pyruvate dehydrogenase kinase 4 (PDK4)/pyruvate dehydrogenase complex (PDC) axis [11]. In response to hypoxia, PDK4 phosphorylates the E1α subunit of the PDC to inactivate it [12, 13]. The elevation of PDK4 in hypoxia therefore decreases the amount of glucose-derived acetyl CoA available to fuel the tricarboxylic acid (TCA) cycle, increasing metabolic efficiency in hypoxia and preserving cell survival, which is essential for EC participation in angiogenesis [6]. The role of the PDK4/PDC axis in EC angiogenesis and the effect of diabetes is relatively unexplored.

Reconstituted high-density lipoproteins (rHDL) have been shown to rescue high glucose-impaired angiogenesis in vitro and in vivo in diabetic models [14]. However, its ability to correct EC metabolic responses to hypoxia in diabetes is unknown. Accordingly, the objectives of this study were to investigate the importance of the PDK4/PDC axis in diabetes-impaired angiogenesis in ECs in vitro and in vivo in a murine model of diabetic wound healing, and determine the ability of rHDL to correct this pathway.

**Materials And Methods**

**Preparation of Discoidal rHDL**

Apolipoprotein A-I (apoA-1) was reconstituted in buffer (100mM Tris, 3M Guanidine HCl, pH 8.2), and dialysed against Tris-buffered saline (TBS). ApoA-I was complexed with 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC) to form discoidal HDL (rHDL). rHDL was dialysed against TBS and
phosphate-buffered saline (PBS). rHDL was filtered, and the concentration of apoA-I determined using a Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Life Technologies). rHDL was stored under N₂ gas [14].

**Animal Studies**

All procedures were conducted with ethical approval from the SAHMRI Animal Ethics Committee (#SAM301) and conformed to the Guide for the Care and Use of Laboratory Animals (NIH). In specific pathogen free housing in cages of four, male 6-week-old C57Bl/6J mice (Jackson Laboratory, USA) were rendered diabetic two weeks prior to surgery by a bolus intraperitoneal injection of streptozotocin (165µg/g). One-week post-injection, hyperglycaemia was confirmed using the Accu-CHEK Performa Blood Glucometer. A blood glucose level of 15.0mmol/L or above was considered diabetic.

**Murine Wound Healing Model**

The wound healing model was conducted as previously described [14, 15]. Briefly, under inhalation isoflurane (3%) anaesthesia, two full thickness wounds were placed on the back flanks and secured open using sutured on silicon splits. For each mouse, one wound received topical rHDL (50µg/wound/day) or PBS (vehicle control), applied daily. A dressing (Opsite™) was applied, and digital images and wound area measurements were taken daily. Wound blood perfusion was determined using laser Doppler imaging. After completion of each time point, mice were humanely killed using an overdose of isoflurane anaesthesia (5%), followed by cardiac puncture, and the wound area excised.

**Immunohistochemistry**

Wound tissue was probed for CD31 to detect neovessels. After tissue processing and sectioning, slides were boiled for 20min in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0) for antigen retrieval. Slides were blocked for 4h with 10% goat serum. Slides were incubated with 1° antibody for CD31 (ab28364, Abcam) at 1:25 in PBST overnight at 4°C, then incubated with HRP-conjugated goat anti-rabbit 2° antibody at 1:200 in PBST for 2h at room temperature. Positively stained neovessels were detected using a DAB Peroxidase Substrate Kit (Vector Labs), and counterstained with haematoxylin. Staining was quantified using ImageJ (NIH).

**Cell Culture and Treatments**

Human coronary artery ECs (HCAECs; Cell Applications Inc.) were cultured in MesoEndo (Cell Applications Inc.) and used at passages 3 to 5. Cells were seeded at 1x10⁵ cells/well, and cultured for 24h at 37°C in 5% CO₂ prior to any treatment. HCAECs were treated with rHDL (20µmol/L, equivalent to 0.6mg/mL; final apoA-I concentration) or PBS control for 18h, then exposed to MesoEndo media with a baseline glucose concentration of 5.5mmol/L for normal glucose conditions, or supplemented with D-glucose to a final concentration 25mmol/L for high glucose conditions for 72h. Media was replenished every 24h. HCAECs were exposed to either normoxia or hypoxia (1.2% O₂ balanced with N₂) for 6h.

**siRNA-mediated PDK4 Knockdown**
HCAECs were seeded at 1.5x10⁵ cells/well, and cultured for 24h at 37°C. Media was replaced with 500μL of Opti-MEM, and 50nM siRNA for PDK4 or a scrambled control (Millenium Science Australia) was added in 300μL Opti-MEM containing 2.5% Lipofectamine 3000 (Thermo Fisher Scientific). Media was replaced after 6h.

**Cellular Metabolic Phenotype**

The metabolic phenotype of pre-treated HCAECs was assessed using the Seahorse Bioanalyzer Cellular Metabolic Phenotype Assay (Agilent). After 48h glucose exposure, HCAECs were re-seeded across two 96-well microplates at 2x10⁴ cells/well in the corresponding glucose media. 24h later, media was changed to assay media and one microplate was run immediately (normoxia) whilst the second microplate was exposed to hypoxia for 6h. Immediately prior to running each plate, several empty wells were seeded with un-treated cells (2x10⁴ cells/well) to act as an interplate control. Data from each well were normalized to total cell number using the CyQUANT Cell Proliferation Assay (Thermo Fisher Scientific).

**Lactate Assay**

Lactate was detected using the Colorimetric/Fluorometric L-Lactate Assay Kit (ab65330, Abcam). Cell media samples were diluted 1:20 in Assay Buffer, and combined with the enzyme and substrate reaction mix to generate colour, which was measured at 450nm using an iMark Microplate Absorbance Reader (Bio-Rad).

**Matrigel Tubulogenesis Assay**

Pre-treated HCAECs were seeded at 1x10⁵ cells/mL on polymerized growth factor-reduced Matrigel (BD Biosciences), then incubated at 37°C in normoxia or hypoxia for 6h. Wells were imaged at 5X magnification under light microscopy. Tubule and branch point number was determined using ImageJ (NIH).

**Boyden Chamber Migration Assay**

Pre-treated HCAECs were seeded at 2x10⁵ cells/mL in Boyden Chamber transwells in Opti-MEM (Thermo Fisher Scientific) containing recombinant VEGFA protein (10ng/mL), then incubated for 18h. Membranes were excised, stained with DAPI and migrated cells imaged at 10X magnification using fluorescence microscopy. Cell number was determined using ImageJ (NIH).

**RNA Expression**

Tissues were homogenised at 6000 x g in 500μL of TRI reagent using a Precellys homogeniser. For treated HCAECs, cells were washed with ice-cold PBS and scraped in 250μL TRI reagent. Following cell lysis, total RNA was isolated [16]. 500ng of RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad). Quantitative real-time PCR was performed for human or murine PDK4/Pdk4, human β2-microglobulin (B2M), and murine 36B4 (Supplementary Table 1 for primer sequences) in HCAECs or wound tissue. Relative changes in gene expression were normalized using the ΔΔCt method to human B2M or murine 36B4.
Protein Expression

Tissues were homogenised at 6000 x g in 150µL of radioimmunoprecipitation (RIPA) buffer for western blot analysis or Cell Extraction Buffer for ELISA analysis using a Precellys homogeniser. For treated HCAECs, cells were washed with ice-cold PBS and scraped in 100µL RIPA buffer. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Life Technologies). Whole-cell protein extracts were probed with antibodies for various targets. Even protein loading was confirmed by probing for α-tubulin (Supplementary Table 2 for antibody details). Human and murine PDK4 expression was measured by ELISA (ab126582 and ab215544, Abcam), as was human HIF-1α (RDSDYC19352, R&D Systems).

Chromatin Immunoprecipitation Assay

Following cell treatments, proteins and DNA were crosslinked by addition of 37% formaldehyde (final concentration of 1%). After 10min, 1M ice-cold glycine was added. Cells were washed and scraped in ChIP lysis buffer. DNA was sonicated to 300bp fragments. Chromatin samples were pre-cleared by incubation with ChIP-grade protein G magnetic beads for 2h at 4°C. Samples were incubated with an antibody for FOXO1 or an IgG Normal Rabbit control (Supplementary Table 2 for antibody details) overnight at 4°C. Protein was immunoprecipitated using the protein G magnetic beads, washed 3x with low salt buffer and 1x with high salt buffer before elution of DNA. Crosslinks were reversed with 5M NaCl, and Proteinase K. DNA was purified using a QIAquick PCR clean-up kit (Qiagen), and qPCR performed to amplify a region of the PDK4 promoter region (See Supplementary Table 1 for primer sequences).

Statistical Analysis

Data are expressed as mean ± SEM. Within experiments, each experimental condition was performed in triplicate. Each whole experiment was then completed at least three times. A two-way ANOVA was used when comparing data across multiple time points or when comparing data spanning three or more conditions (Tukeys posthoc test of significance). Differences between treatment groups were calculated using a Student’s t-test or one-way ANOVA (Tukeys posthoc test of significance). Paired t-tests were used when comparing data points from the same mice. Significance was set at \( P < 0.05 \).

Results

Diabetes impairs metabolic reprogramming responses to wound ischemia and is rescued by rHDL in diabetic mice

Mice receiving STZ injections had significantly higher blood glucose levels than non-diabetic control mice (Non-diabetic: 10.1 ± 1.3 versus Diabetic: 25.7 ± 6.4 mmol/L, \( P < 0.0001 \)) and lower body weights (Non-diabetic: 24.5 ± 1.8 versus Diabetic: 22.9 ± 2.1 g, \( P < 0.05 \)) at the time of wound surgery (Supplementary Table 3). At day 1 post-wounding in non-diabetic mice there was an induction of PDK4 protein levels (3.0 ± 1.2 to 9.3 ± 2.0 ng/mL, 207% increase, \( P < 0.05 \), Fig. 1A). This induction did not occur in diabetic mice.
However, topical rHDL restored the induction of PDK4 protein expression at day 1 post-wounding in diabetic wounds (4.4 ± 1.0 to 7.4 ± 1.6 ng/mL, 68% increase, *P* < 0.05, Fig. 1A). Consistent with this, at day 3 post-wounding rHDL-treated wounds exhibit enhanced pPDC in diabetic mice (63 ± 8 to 167 ± 39%, 165% increase, *P* < 0.01, Fig. 1B-C). In non-diabetic mice, wound *Pdk4* mRNA levels were also significantly increased at day 1 post-wounding (100 ± 43 to 288 ± 75%, 188% increase, *P* < 0.05, Fig. 1D). Diabetes impaired this induction, with *Pdk4* mRNA expression significantly reduced in Day 1 diabetic wounds (288 ± 75 to 127 ± 29%, 56% decrease, *P* < 0.01, Fig. 1D). In diabetic mice, topical application of rHDL enhanced *Pdk4* mRNA levels at day 3 post-wounding (22 ± 5 to 40 ± 9%, 82% increase, *P* < 0.05, Fig. 1D and inset).

**rHDL increases wound neovascularization and rescues diabetes-impaired healing in vivo**

Blood flow perfusion of the wound was determined as a ratio of rHDL:PBS using laser Doppler imaging. In diabetic mice rHDL significantly increased wound blood flow perfusion 3 days post-wounding (99 ± 11 to 140 ± 35%, 41% increase, *P* < 0.05, Fig. 2A-B). The PBS-treated wounds of diabetic mice contained fewer CD31⁺ neovessels compared to the PBS-treated wounds of non-diabetic mice (36 ± 4 to 21 ± 3 neovessels, 42% decrease, *P* < 0.05, Fig. 2C-D). rHDL treatment rescued this impairment in diabetic mice (21 ± 3 to 34 ± 4 neovessels, 62% increase, *P* < 0.05). The PBS-treated wounds of diabetic mice exhibited reduced rates of wound closure, reaching significance at Day 8 (60 ± 2 to 48 ± 4% wound closure, 20% decrease, *P* < 0.05, Fig. 2E-F), compared to non-diabetic mice with PBS-treated wounds. Topically applied rHDL significantly increased the rate of wound closure in diabetic mice across all timepoints (increases ranging from 31.9-154.9%, *P* < 0.01, *P* < 0.0001), compared to diabetic PBS-treated wounds.

**High glucose impairs metabolic reprogramming responses to hypoxia, which is restored by rHDL in vitro**

We next studied how the PDK4/PDC axis was affected in high glucose and hypoxia *in vitro*, and its regulation by rHDL. Under normal glucose conditions at 5mM, hypoxia exposure increased EC *PDK4* mRNA expression (100 ± 5 to 165 ± 16%, 65% increase, *P* < 0.05, Fig. 3A). However, exposure to high glucose at 25mM impaired a further stepwise induction of *PDK4* in response to hypoxia. An additional stepwise induction is essential to adequately reduce the activity of the PDC to protect against both the effects of hypoxia and high glucose. However, preincubation with rHDL in high glucose restored this response to hypoxia, inducing *PDK4* mRNA (176 ± 15 to 240 ± 17%, 37% increase, *P* < 0.05) and PDK4 protein (7.4 ± 0.6 to 10.8 ± 0.8 ng/mL, 44% increase, *P* < 0.05, Fig. 3B), compared to the PBS high glucose control. Consistent with these findings, in normal glucose conditions, PDC phosphorylation (pPDC) increased in response hypoxia (130 ± 11 to 213 ± 25%, 64% increase, *P* < 0.01, Fig. 3C). Exposure to high glucose in hypoxia did not induce a further elevation in pPDC levels, however, rHDL corrected this, and promoted higher pPDC levels (100 ± 6 to 170 ± 22%, 70% increase, *P* < 0.01).
We next aimed to determine whether this change in regulation of the PDK4/PDC axis was associated with changes in cellular oxygen consumption rate (OCR), a measure of mitochondrial respiration [17]. In normoxia, high glucose exposure increased OCR (100 ± 3 to 119 ± 3%, 19% increase, \( P<0.01 \), Fig. 3E), compared to normal glucose. However, rHDL prevented this and kept OCR at baseline levels. Overall, hypoxia lowered the OCR across all treatments when compared to normoxia groups. However, high glucose exposure in hypoxia increased OCR (72 ± 4 to 86 ± 3%, 19% increase, \( P<0.05 \)), compared to normal glucose control. rHDL prevented this high glucose-induced elevation of the OCR in hypoxia, significantly lowering the OCR (86 ± 3 to 72 ± 3%, 16% decrease, \( P<0.05 \)), compared to PBS control cells in high glucose and hypoxia. rHDL treatment significantly decreased extracellular lactate under high glucose conditions (96 ± 3 to 78 ± 4%, 19% decrease, \( P<0.01 \), Fig. 3F), compared to the PBS control.

**rHDL rescues high glucose-impaired EC function in vitro**

In hypoxia, high glucose exposure impaired tubule (117 ± 4 to 85 ± 9%, 28% decrease, Fig. 4A-B) and branch point formation (128 ± 2 to 95 ± 7%, 26% decrease, Fig. 4AC) \( P<0.05 \) for both, compared to cells in normal glucose and hypoxia. rHDL rescued this impairment, increasing tubule (85 ± 7 to 136 ± 10%, 60% increase, \( P<0.001 \)) and branch points (95 ± 7 to 169 ± 4%, 78% increase, \( P<0.0001 \)). High glucose impaired migration in normoxia (100 ± 6 to 60 ± 7, 40% decrease, \( P<0.01 \), Fig. 4D-E) and hypoxia (107 ± 7 to 65 ± 10, 39% decrease, \( P<0.01 \)), compared to the respective controls. In hypoxia, rHDL rescued migration, elevating the number of migrated cells (65 ± 10 to 100 ± 6%, 55% increase, \( P<0.05 \)), compared to the PBS control.

**PDK4 knockdown attenuates the pro-angiogenic effects of rHDL**

High glucose exposure impaired tubule formation in the non-transfected (100 ± 3 to 81 ± 6%, 19% decrease, \( P<0.05 \), Fig. 5B), scrambled siRNA control (97 ± 3 to 71 ± 4%, 27% decrease, \( P<0.01 \)) and PDK4 siRNA ECs (72 ± 7 to 63 ± 6%, 12% decrease, \( P<0.05 \)), when compared to their respective normal glucose controls (Fig. 5A-B). PDK4 knockdown impaired tubule formation in normal glucose PBS cells, compared to both the non-transfected (100 ± 3 to 72 ± 7%, 28% decrease, \( P<0.001 \)) and scrambled siRNA controls (97 ± 3 to 72 ± 7%, 26% decrease, \( P<0.01 \)). rHDL treatment rescued high glucose-impaired tubulogenesis in both non-transfected (81 ± 6 to 121 ± 3%, 50% increase, \( P<0.0001 \)) and scrambled siRNA controls (71 ± 4 to 108 ± 6%, 52% increase, \( P<0.0001 \)), but not in the cells deficient in PDK4.

**High glucose suppresses the induction of HIF-1α in response to hypoxia, rHDL has no effect on HIF-1α, but reduces PHD1, PHD2, and PHD3**
HIF-1α has been demonstrated to play a role in regulation of PDK4 transcription [12]. Expression of HIF-1α was significantly increased with hypoxia exposure across all conditions ($P<0.0001$, Fig. 6A). High glucose exposure impaired expression of HIF-1α under hypoxic conditions (83 ± 6 to 62 ± 6 pg/mL, 29% decrease, $P<0.01$), compared to normal glucose in hypoxia. rHDL treatment had no effect on HIF-1α expression in either normal or high glucose. The role of the PHD proteins (1, 2 and 3) is primarily to target HIF-1α for degradation in normoxia, with their expression downregulated in hypoxia to allow accumulation of HIF-1α [18]. The PHD proteins also play roles in other transcription pathways [10, 19, 20]. Despite a lack of change in HIF-1α, rHDL decreased expression of PHD1 in normoxia and high glucose conditions (112 ± 12 to 83 ± 6%, 26% decrease, $P<0.05$, Fig. 6B) but this suppression was not seen in hypoxia (Fig. 6C). Whilst there were no changes in PHD2 in normoxia (Fig. 6D), rHDL decreased PHD2 protein levels under high glucose conditions and in hypoxia, compared to PBS high glucose controls (99 ± 10 to 73 ± 6%, 26% decrease, $P<0.05$, Fig. 6E). In normoxia, PHD3 expression was decreased with high glucose (100 ± 4 to 80 ± 7%, 20% decrease, $P<0.05$, Fig. 6F). rHDL treatment further reduced PHD3 expression in high glucose in both normoxia (80 ± 7 to 54 ± 5%, 32% decrease, $P<0.01$, Fig. 6G) and hypoxia (65 ± 6 to 46 ± 5%, 29% decrease, $P<0.05$), compared to PBS high glucose-treated cells.

**rHDL reduces phosphorylation of FOXO1 and enhances its transcription factor activity in high glucose and hypoxia**

In the absence of a change in HIF-1α with rHDL, we next examined FOXO1, a transcription factor which interacts with the PDK4 promoter [21] and plays a role in the regulation of angiogenesis [22]. In hypoxia under normal glucose conditions, FOXO1 phosphorylation was increased (100 ± 4 to 149 ± 18%, 49% increase, $P<0.05$, Fig. 7A-B), compared to the normoxia control. In hypoxia under high glucose, rHDL treatment decreased the phosphorylation of FOXO1 (193 ± 13 to 138 ± 15%, 29% decrease, $P<0.05$), compared to PBS and high glucose-treated cells, returning it to the level observed under normal glucose conditions. A chromatin immunoprecipitation assay (ChIP) assessed FOXO1 interaction with a known site within the PDK4 promoter region [23, 24]. High glucose exposure reduced the amount of FOXO1-bound to the PDK4 promoter compared to the normal glucose control (100 ± 4 to 8.7 ± 1%, 91% decrease, $P<0.0001$). Hypoxia exposure significantly reduced FOXO1-bound PDK4 promoter compared to the normoxia control (100 ± 4 to 39 ± 5%, 62% decrease, $P<0.01$). rHDL treatment under high glucose and hypoxic conditions enhanced the amount of FOXO1-bound PDK4 promoter sequence (34 ± 3 to 61 ± 13%, 79% increase, $P<0.05$).

**Discussion**

Diabetes impairs ischemia-driven angiogenesis, which contributes to the development of diabetic vascular complications [25]. EC metabolic reprogramming in response to hypoxia is an important part of preserving EC function. The PDK4/PDC axis is central to the regulation of metabolic reprogramming [13], disruption in this axis may contribute to the impairment of angiogenesis in diabetes. We postulated rHDL corrects this impairment as it has pro-angiogenic effects in high glucose and diabetic mouse models [14].
We report the following important findings: (1) Diabetes impairs metabolic reprogramming responses to wound ischemia in vivo (2) rHDL rescues this, while also rescuing impaired wound healing and enhancing neovascularization; (3) high glucose impairs EC metabolic reprogramming responses to hypoxia, with rHDL incubation returning the regulation of the PDK4/PDC axis to that seen in normal glucose; (4) rHDL rescues high glucose-impaired in vitro angiogenic functions, and siRNA knockdown of PDK4 ameliorates these effects of rHDL on angiogenesis. Finally, (5) the mechanism underlying the metabolic effects of rHDL may be elicited by activation of FOXO1 activity and binding to the PDK4 promoter (Fig. 8). Taken together, we show diabetes impairs metabolic reprogramming responses to hypoxia and demonstrate a new mechanism by which rHDL rescues diabetes-impaired angiogenesis.

We observed a striking impairment to the induction of PDK4 and pPDC in diabetic mice in the early stages post-wounding. While several studies have highlighted the importance of glycolysis in EC angiogenesis [9, 27], few have examined suppression of mitochondrial respiration as an oxygen-conserving mechanism in hypoxia-driven angiogenesis. This result indicates that control of mitochondrial respiration is perturbed in diabetic wound tissue, and may contribute to impaired neovascularisation.

The known effect of diabetes on PDK4 expression and activity varies depending upon the tissue. For example, PDK4 mRNA expression is elevated in muscle tissue from diabetic mice, indicating diabetes causes impaired glucose utilisation [25]. Other studies have shown that general cellular metabolism is perturbed in different ways across diabetic tissues [26]. This variation is expected, since different tissues display unique metabolic phenotypes that support their function. Our findings highlight the clear effect of diabetes on metabolic reprogramming in murine wound tissue, but also the importance of the PDK4/PDC axis in the early response to wound ischaemia. A strength of this study is the measurement of key metabolic in vivo markers in separate cohorts of mice over time post-wound ischemia, providing a clear demonstration of their expression patterns through the different phases of healing. We identified that the axis plays a lesser role in the later stages of wound healing. The tissue-specific and temporal elements must both be considered in the translation of these findings.

rHDL enhanced PDK4 and pPDC levels in wound tissues of diabetic mice, and enhanced both neovascularization and healing, suggesting a potential association between the PDK4/PDC axis and neovascularisation. Our in vitro findings in HCAECs confirmed this as siRNA deletion of PDK4 inhibited tubulogenesis.

We demonstrated that hypoxia exposure increased PDK4 mRNA expression and pPDC, highlighting the importance of this mechanism in the EC hypoxia response. Importantly, exposure to high glucose in hypoxia did not elicit an additional stepwise induction in PDK4 expression and pPDC and increased mitochondrial respiration. This demonstrated that high glucose negatively affects the cells’ ability to suppress respiration. In parallel, high glucose negatively impacted angiogenic functions in response to hypoxia, suggesting that aberrant metabolic responses to hypoxia contribute to the impairment of angiogenesis in high glucose.
Preincubation with rHDL rescued high glucose-impaired metabolic reprogramming responses to hypoxia. In high glucose and hypoxia, rHDL elevated PDK4 and PDC phosphorylation, reduced mitochondrial respiration and rescued in vitro angiogenic functions. When PDK4 was knocked down these effects of rHDL on angiogenesis were attenuated, demonstrating the importance of PDK4 in mediating the pro-angiogenic properties of rHDL. Overall, our data demonstrate that rHDL corrects impaired metabolic reprogramming responses to hypoxia and rescues impaired angiogenesis.

The question of how rHDL elicits changes in PDK4 transcription was central to this study. In the absence of an effect on the main hypoxia transcription factor HIF-1α, we investigated the transcription factor FOXO1 as it is known to interact with the PDK4 promoter region [21]. When FOXO1 is phosphorylated, it is excluded from the nucleus and its transcriptional activity is abrogated [21, 31, 32]. A role for FOXO1 in angiogenesis and EC function has previously been identified. Wilhelm et al. demonstrated that deletion of FOXO1 in mice caused an increase in vessel sprouting [22]. Conversely, its overexpression severely restricted angiogenesis and led to vessel thinning. The angiogenic role of FOXO1 in response to hypoxia was not examined. We observed that phosphorylation of FOXO1 was increased under hypoxic conditions, indicating a decrease in its nuclear activity. With high glucose exposure, phosphorylation was further increased, potentially pushing its activity below acceptable levels which may explain the impairment to PDK4 expression. rHDL significantly decreased FOXO1 phosphorylation, restoring its levels to those seen in the normal glucose control. This finding was supported by ChIP that showed rHDL increased binding of FOXO1 to the PDK4 promoter, thereby presenting a mechanism to explain our observed increases in PDK4 expression by rHDL (Fig. 8).

In conclusion, we have demonstrated that diabetes impairs metabolic reprogramming and angiogenic responses to hypoxia, which are rescued by rHDL in vitro and in vivo. These findings provide further mechanistic support for the effects of rHDL on diabetes-impaired angiogenesis and has significant implications for the translation of HDL, an endogenous agent, as a that improves tissue regeneration in diabetes.

Declarations

Ethics approval and consent to participate: All procedures were conducted with ethical approval from the SAHMRI Animal Ethics Committee (#SAM301) and conformed to the Guide for the Care and Use of Laboratory Animals (NIH).

Consent for publication: Not applicable

Availability of data and materials: Not applicable

Competing interests: The authors have no competing interests to disclose.

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Author contributions: C.A.B and K.R.P conceived and designed this study. K.R.P conducted the experiments with the support of J.T.M.T. K.R.P analysed the data and with C.A.B. wrote the manuscript. P.J.P and J.T.M.T. helped interpret the data and provided intellectual input into the preparations of the manuscript.

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**Figures**
Diabetes impairs metabolic reprogramming responses to wound ischemia and rHDL rescues this impairment in diabetic mice

Two full-thickness wounds were created on non-diabetic and diabetic C57Bl/6J mice. Mice received daily topical applications of rHDL (50 μg/wound) or PBS (vehicle). Wound tissue was collected from non-
diabetic (white and grey circles) and diabetic (red and blue triangles) mice at days 0, 1, 3, and 10 post-wound creation. (A) PDK4 protein expression at days 0, 1 and 3 post-wounding was measured by ELISA (n=4, 9, 12) *P<0.05, **P<0.01 vs controls by two-way ANOVA. (B-C) Phosphorylation of the PDC at days 0, 1, and 3 post-wounding was measured by western blotting (n=5, 7, 10) **P<0.01 vs. PBS control by two-way ANOVA. (D) Pdk4 mRNA expression was measured using qRT-PCR. Gene expression was normalized using the ΔΔCt method to murine 36B4. (n=8, 13) *P<0.05, **P<0.01 vs. control by two-way ANOVA. *P<0.05 by paired t-test. Data are expressed as mean±SEM.
Figure 2

**rHDL increases neovascularization and rescues diabetes-impaired wound healing in vivo.**

Two full-thickness wounds were created on non-diabetic and diabetic C57Bl/6J mice. Mice received daily topical applications of rHDL (50 µg/wound) or PBS (vehicle). (A-B) rHDL:PBS wound blood flow perfusion ratio was determined using laser Doppler perfusion imaging; images represent high (red) to low (blue)
blood flow from day 1-9 in non-diabetic (black circles) and diabetic (blue circles) mice. *P<0.05 vs. PBS control by paired t-test. (C-D) Capillaries were identified in wound sections using immunohistochemistry for CD31. Photomicrographs represent wounds stained for CD31 (stained brown, denoted by arrows). Scale bars, 200 μm. *P<0.05 vs. PBS control by one-way ANOVA (n=9, 14) (E-F) Wound area was calculated from the average of three daily diameter measurements along the x-, y-, and z-axes. Wound closure is expressed as a percentage of initial wound area at day 0. *P<0.05, **P<0.01, ***P<0.001, *****P<0.0001 vs. relevant controls by two-way ANOVA. ^P<0.05 vs. non-diabetic PBS-treated wound by two-way ANOVA. (n=12). Non-diabetic PBS-treated wounds (black circles), non-diabetic rHDL-treated wounds (grey circles), diabetic PBS-treated wounds (red triangles), diabetic rHDL-treated wounds (blue triangles). Data are expressed as mean±SEM.
Figure 3

High glucose impairs metabolic reprogramming responses to hypoxia in ECs and rHDL restores this impairment in vitro

HCAECs were incubated with rHDL (20 µM) or PBS (vehicle control) for 18 h, then exposed to normal (5 mmol/L) or high (25 mmol/L) glucose conditions for 72 h. Following this, cells were exposed to
normoxia or hypoxia (1.2% O$_2$) for 6 h. (A) $PDK4$ mRNA expression was measured using qRT-PCR and normalized using the $\Delta\Delta$Ct method to human $B2M$, (n=9). (B) $PDK4$ protein expression was measured using an ELISA, (n=4, 6). (C-D) Phosphorylation of the PDC at serine 293 was measured using western blotting densitometry, with data expressed as the ratio of pPDC(S293):Total PDC, and normalized to $\alpha$-tubulin, (n=10). *$P<0.05$, **$P<0.01$, ***$P<0.001$ by two-way ANOVA. (E) Oxygen consumption of treated cells was measured using the Seahorse Bioanalyser system, with data from each well normalized to cell number (n=9, 12). *$P<0.05$, **$P<0.01$, ***$P<0.001$ by two-way ANOVA. ^^^$P<0.001$, ^^^$P<0.0001$ vs. normoxia control by two-way ANOVA. (F) Lactate in the media of hypoxia-exposed and treated cells was measured using a colorimetric assay, (n=5, 6). **$P<0.01$ by unpaired t-test. 5 mM glucose, PBS (white circles), 5 mM glucose, rHDL (grey circles), 25 mM glucose, PBS (red triangles), 25 mM glucose, rHDL (blue triangles). Data are expressed as mean±SEM.
**Figure 4**

rHDL rescues high glucose-impaired EC function *in vitro*.

HCAECs were incubated with rHDL (20 µM) or PBS (vehicle control) for 18 h, then exposed to normal (5 mmol/L) or high (25 mmol/L) glucose conditions for 72 h. (A) Treated cells were then seeded on Matrigel and exposed to normoxia or hypoxia (1.2% O²) for 6 h. Representative images of tubules from all
conditions. Matrigel tubules (B) and branch points (C) were imaged using light microscopy and quantified using ImageJ software (n=9). Treated cells were seeded in wells containing a permeable membrane to measure cell migration. (D) Representative images from all conditions. Membranes were imaged using fluorescence microscopy and quantified (E) using ImageJ software (n=9). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 by two-way ANOVA. 5 mM glucose, PBS (white circles), 5 mM glucose, rHDL (grey circles), 25 mM glucose, PBS (red triangles), 25 mM glucose, rHDL (blue triangles). Data are expressed as mean±SEM.

Figure 5

PDK4 knockdown attenuates the pro-angiogenic effects of rHDL
HCAECs were treated with PDK4-specific siRNA (50 nM) or a scrambled control for 6 h, then incubated with fresh media for 48 h. HCAECs were then incubated with rHDL (20 µM) or PBS (vehicle control) for 18 h, then exposed to normal (5 mmol/L) or high (25 mmol/L) glucose conditions for 72 h. Treated cells were then seeded on Matrigel and exposed to normoxia or hypoxia (1.2% O\textsubscript{2}) for 6 h. (A) Representative light microscopy images of tubules from the siScrambled and siPDK4 conditions. (B) Matrigel tubules were imaged and quantified using ImageJ software (n=10). *P<0.05, **P<0.01, ****P<0.0001 vs. controls by two-way ANOVA. ###P<0.001 vs. non-transfected control by two-way ANOVA. ^^P<0.01 vs. siScrambled control by two-way ANOVA. 5 mM glucose, PBS (white circles), 5 mM glucose, rHDL (grey circles), 25 mM glucose, PBS (red triangles), 25 mM glucose, rHDL (blue triangles). Data are expressed as mean±SEM.
Figure 6

High glucose suppresses the induction of HIF-1α in response to hypoxia, rHDL has no effect on HIF-1α, but reduces expression of PHD1, PHD2, and PHD3

HCAECs were incubated with rHDL (20 µM) or PBS (vehicle control) for 18 h, then exposed to normal (5 mmol/L) or high (25 mmol/L) glucose conditions for 72 h. Following this, cells were exposed to normoxia.
or hypoxia (1.2% O$^2$) for 6 h. (A) Whole-cell HIF-1α protein expression was measured using an ELISA (n=14). **$P<0.01$, ***$P<0.001$ vs. controls. ^^^^$P<0.0001$ vs. normoxia controls. All by two-way ANOVA. Whole-cell protein expression of (B-C) PHD1 (n=12), (D-E) PHD2 (n=5), and (F-G) PHD3 (n=14) was measured using western blotting densitometry, with data normalized to α-tubulin. *$P<0.05$, **$P<0.01$ by unpaired t-test. 5 mM glucose, PBS (white circles), 5 mM glucose, rHDL (grey circles), 25 mM glucose, PBS (red triangles), 25 mM glucose, rHDL (blue triangles). Data are expressed as mean±SEM.
Figure 7

**rHDL increases FOXO1 activity and binding to the PDK4 promoter in high glucose and hypoxia.**

HCAECs were incubated with rHDL (20 µM) or PBS (vehicle control) for 18 h, then exposed to normal (5 mmol/L) or high (25 mmol/L) glucose conditions for 72 h. Following this, cells were exposed to normoxia or hypoxia (1.2% O₂) for 6 h. (A) Representative Western blot images of phosphorylated FOXO1 (pFOXO1)
and total FOXO1 levels (B) Graphed densitometry analyses of pFOXO1:Total FOXO1, with data normalized to α-tubulin (n=5). *P<0.05 by two-way ANOVA. (C) A schematic showing the FOXO1 binding site sequence (underlined) in the PDK4 gene promoter region that was targeted by the chromatin immunoprecipitation assay (ChIP). (D) FOXO1 binding to this known site in the PDK4 promoter was measured using ChIP (n=3). *P<0.05, **P<0.01, ****P<0.0001 vs. controls by two-way ANOVA. ^^^^^P<0.0001 vs 5mM glucose, PBS control by two-way ANOVA. 5 mM glucose, PBS (white circles), 5 mM glucose, rHDL (grey circles), 25 mM glucose, PBS (red triangles), 25 mM glucose, rHDL (blue triangles). Data are expressed as mean±SEM.

Figure 8

Proposed mechanism of impairment to the PDK4/PDC axis by diabetes and its regulation by rHDL.
Diabetes impairs the induction of the PDK4/PDC axis in response to hypoxia, which is associated with aberrant metabolic responses and an impairment to EC function. rHDL decreases expression of the PHD proteins, which may affect phosphorylation of FOXO1. Decreased phosphorylation of FOXO1 leads to increased transcription factor activity, which increases binding of FOXO1 to the PDK4 promoter region and the transcription of PDK4. Increased PDK4 is associated with an increase in inactivation of the PDC, which would lead to a decrease in the amount of glucose-derived acetyl CoA available to fuel the TCA cycle. Overall mitochondrial respiration is decreased, improving cell survival in high glucose and hypoxia and preserving hypoxia-induced angiogenesis.

**Supplementary Files**

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