The role of hypoxia inducible factor 1α in diabetic cardiomyopathy

Romana Bohuslavova¹
Email: romana.bohuslavova@img.cas.cz

Frantisek Kolar²
Email: kolar@biomed.cas.cz

David Sedmera²,³
Email: david.sedmera@lf1.cuni.cz

Lada Skvorova¹
Email: lada.skvorova@img.cas.cz

Frantisek Papousek²
Email: papousek@biomed.cas.cz

Jan Neckar²
Email: jneckar@mcw.edu

Gabriela Pavlinkova¹§
Email: gpavlinkova@img.cas.cz

¹Institute of Biotechnology AS CR, Prague, Czech Republic
²Institute of Physiology AS CR, Prague, Czech Republic
³Institute of Anatomy, First Faculty of Medicine, Charles University, Prague, Czech Republic

§Corresponding author: Laboratory of Molecular Pathogenetics, Institute of Biotechnology AS CR, v.v.i., Vídenská 1083, Prague 4, CZ-142 20, Czech Republic
Abstract

Background
Diabetic cardiomyopathy is associated with a number of functional and structural pathological changes, including systolic dysfunction, myocardial remodeling, cardiac apoptosis, inflammation, and left ventricular abnormalities. The primary cause of diabetic cardiomyopathy is hyperglycemia, the metabolic hallmark of diabetes. Recent studies have shown that hyperglycemia suppresses hypoxia-inducible factor (HIF)-1α protein stability and function. The aim of this study was to analyze the functional role of HIF-1α in the early stages of diabetic cardiomyopathy.

Methods
Diabetes was induced by streptozotocin in wild type (Wt) and heterozygous Hif1α knock-out (Hif1α+/−) mice. Echocardiographic evaluations of left ventricle functional parameters, gene expression profiling by qPCR, Western blot analysis, and cardiac histopathology assessments were performed in age-matched groups, diabetic, and non-diabetic Wt and Hif1α+/− mice.

Results
Five weeks after diabetes was established, a significant systolic dysfunction was evident in diabetic Hif1α+/− but not in diabetic Wt mice. A significant decrease in left ventricle (LV) fractional shortening was detected in diabetic Hif1α+/− compared to diabetic Wt mice. The combinatory effects of the haploinsufficiency genotype and diabetes affected the gene expression profile of the heart, including reduced vascular endothelial growth factor A (Vegfa) expression. The number of apoptotic cells was moderately increased in the diabetic Wt hearts but not in the diabetic Hif1α+/− hearts. Heterozygosity for Hif1α stimulated adverse cardiac remodeling in the diabetic heart. The diabetic Hif1α+/− LV showed increased protein
levels of Collagen 1 and changes in Connexin 43 distribution compared to the diabetic Wt heart.

**Conclusions**
We have shown a correlation between heterozygosity for *Hif1α* and the activation of adverse functional and structural changes associated with diabetic cardiomyopathy. Our results provide evidence that HIF-1α regulates early cardiac responses to diabetes, and that HIF-1α deregulation may influence the increased risk for diabetic cardiomyopathy.

**Keywords**
Echocardiographic parameters, hypoxia inducible factor 1α, diabetic cardiomyopathy, vascular endothelial growth factor A, heterozygous *Hif1a* knock-out
Background

Both type 1 and type 2 diabetes are characterized by the increased risk of cardiomyopathy and myocardial infarction. Diabetic cardiomyopathy is associated with a number of functional and structural pathological changes, including decreased diastolic compliance, systolic dysfunction, myocardial remodeling, cardiac apoptosis, inflammation, and left ventricular abnormalities. Diabetes is also associated with a majority of risk factors for cardiac failure, such as hypertension, hyperlipidemia, obesity, thrombosis, autonomic neuropathy, endothelial dysfunction, and microvascular pathology [1-3]. Hyperglycemia triggers diabetic tissue damage, including cardiovascular and microvascular complications. Existing evidence suggests that hyperglycemia induces an altered metabolism, the overproduction of reactive oxygen species (ROS), and mitochondrial dysfunction, which are underlying mechanisms behind pathological changes in diabetes [2]. Hypoxia is another important pathophysiological factor associated with diabetic complications.

Transcriptional responses to hypoxia are mediated by hypoxia inducible factor 1 (HIF-1). HIF-1 activates over 800 target genes that are involved in cell proliferation, angiogenesis, glycolytic energy metabolism, and apoptosis [4]. HIF-1 consists of two subunits, HIF-1α, the regulatory subunit, and constitutively expressed HIF-1β. Oxygen tension plays a key role in the regulation of HIF-1α expression, stabilization, and activation [4]. The bulk of this response can be further modulated by growth factor and cytokine dependent signaling pathways [5, 6]. Furthermore, existing evidence indicates that mitochondrial ROS are sufficient enough to initiate the stabilization and activation of HIF-1α, and that treatment with antioxidants prevents HIF-1α protein stabilization [7, 8]. The critical role of Hif1a is demonstrated by the global deletion of HIF-1α, resulting in embryonic lethality due to cardiovascular defects [9]. Hif1a+/− mutants normally survive past embryonic development;
however, *Hif1a* heterozygotes demonstrate impaired responses when challenged with hypoxia after birth [10-12]. Cardiac myocyte-specific HIF-1α gene deletion causes reductions in contractility, vascularization, and alters the expression of multiple genes in the heart during normoxia [13]. These findings point toward the central role of HIF-1α in coordinating the function and energy metabolism in the heart and, together, they have also implications for diseases with impaired oxygen delivery, such as diabetes.

The diabetic environment induces HIF-1α destabilization and it reduces the production and function of the HIF-1α protein [14-17]. Studies using cardiac-specific HIF-1α-overexpressing transgenic mice have shown cardiac protection from diabetic-induced impairment in glucose metabolism and angiogenesis [18]. Furthermore, the loss of HIF-1 activity appears to play a role in the pathogenesis of type 2 diabetes [19, 20]. The aim of this study was to examine HIF-1α function in early cardiac responses to diabetes. We showed that the globally decreased expression of *Hif1a* impaired the abilities to compensate for the initial pathological changes induced by the diabetic environment in the heart. The results demonstrated that the decreased expression of HIF-1α in combination with diabetes affected the heart functions and gene expression profile of the heart.

**Methods**

**Experimental animals**

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). The experimental protocol was approved by the Animal Care and Use Committee of the Institute of Molecular Genetics, Academy of Sciences of the Czech Republic. The experimental mice were housed in a controlled environment (23°C; 12-h light/dark cycle) with free access to water and a standard chow diet.
All experiments were performed with male and female littermate mice that were either wild-type, \( Hif1a^{+/+} \) (Wt) or heterozygous \( Hif1a \) knock-out (\( Hif1a^{+/−} \)) on an FVB background (strain code 207, Charles River). The heterozygous \( Hif1a \) mutants have the \( Hif1a^{tm1jhu} \) mutant allele in which exon 2, encoding the bHLH domain of \( Hif1a \) gene, has been replaced by intragenic deletion with a neomycin resistance (\( neo^R \)) gene [9]. The heterozygous \( Hif1a \) deficient mice showed a partial loss of HIF-1α protein expression levels [21, 22]. Offspring of \( Wt \times Hif1a^{+/−} \) matings were genotyped by PCR, using genomic DNA isolated from tails and amplifying neomycin (\( Neo \)) and \( Hif1a \) exon 2 sequences [9, 23]. Both \( Neo \) (463-bp) and \( Hif1a \) (317-bp) sequences were amplified from DNA of \( Hif1a^{+/−} \) mice, whereas only \( Hif1a \) sequences were amplified from DNA of \( Wt \) mice (\( Hif1a^{+/+} \)), respectively. The sequences of \( Neo \) primers were 5′-ACTGGCTGCTATTGGGCGAAGTG-3′ and 5′-GTAAAGCACGAGGAAGCGGTCAG-3′. The conditions for PCR were 94°C for 30 s, 48°C for 30 s, and 72°C for 30 s, for 40 cycles. The sequences of \( Hif1a \) exon 2 primers were 5′-TGTAGTCTCCTGCTAAAAG-3′ and 5′-GTAAAGCACGAGGAAGCGGTCAG-3′. Conditions for PCR were 94°C for 30 s, 63°C for 30 s, and 72°C for 30 s, for 40 cycles.

Diabetes was induced by 2 intraperitoneal injections of 100 mg/kg body weight of streptozotocin (STZ; Sigma, St. Louis, MO), as described in [24]. The blood glucose levels were measured by glucometer (COUNTOUR TS, Bayer, Switzerland) one week after the last STZ injection. Mice whose blood glucose levels exceeded 13.9 mmol/L were considered diabetic. The mice were analyzed after being diabetic for 5 weeks. The blood glucose levels (mean ± SD) of \( Wt \) and \( Hif1a^{+/−} \) mice were 9.9 ± 0.3 and 10.0 ± 0.3 mmol/L before STZ treatment, and 32.5 ± 1.8 and 30.3 ± 2.0 mmol/L after 5 weeks of diabetes, respectively.
Echocardiography

The echocardiographic evaluation of geometrical and functional parameters of the left ventricle (LV) was performed using the GE Vivid 7 Dimension (GE Vingmed Ultrasound, Horten, Norway) with a 12 MHz linear matrix probe M12L. The animals were anesthetized by the inhalation of 2% isoflurane (Aerrane, Baxter SA) and their rectal temperature was maintained within 36.5 and 37.5°C by a heated table throughout the measurements. For the baseline evaluation, the following diastolic and systolic dimensions of the LV were measured: the posterior wall thickness (PWT_D and PWT_S), anterior wall thickness (AWT_D and AWT_S), and the cavity diameter (LVD_D and LVD_S). From these dimensions, the main functional parameter, fractional shortening (FS) was derived by the following formula: FS [%] = 100 × (LVD_D – LVD_S) / LVD_D.

After the echocardiographic examination, a fluid filled catheter connected to an external transducer (Bpr-02, Experimetria) was introduced into the left carotid artery to measure the blood pressure. The mean blood pressure was averaged from five measurements within a 10-min interval. The hearts were then rapidly excised and dissected into the right ventricle (RV), the LV and the interventricular septum. All ventricular parts were weighed and processed for subsequent analyses.

Western blot

Western blotting was performed using the standard protocol. Dissected LV from the diabetic and non-diabetic hearts were lysed with protease and phosphatase inhibitors to prevent degradation and stored at –80°C until analysis. The protein levels were quantified using the BCA assay. 20 µg of total protein lysates per lane were denatured, resolved using 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% dry milk and incubated overnight with rabbit anti-collagen, type 1 at 1: 1000 dilution
(#203002; MD bioproducts, Zürich, Switzerland), rabbit anti-connexin 43 at 1:6000 (#C6219, Sigma), or rabbit anti-phopho-connexin 43 at 1:1000 (#3511Cell Signaling). After incubation with a horseradish peroxidase–conjugated secondary IgG (Amersham, IL, USA), the blots were developed using the SuperSignal* West Dura Chemiluminescent Substrate (Thermo Scientific, MI, USA). Chemiluminescent signals were captured using an ImageQuant LAS 4000 Imager (GE Healthcare Bio-Sciences AB, Sweden) and analyzed by ImageJ software (http://imagej.nih.gov/ij/download.html). Protein levels were quantified on duplicate blots and were normalized to the loading control ATP5a, mitochondrial membrane marker (#ab140365, Membrane Fraction WB Cocktail, Abcam, Cambridge, USA).

**Quantitative Real-time PCR**

RNA was isolated from the LV of individual diabetic and non-diabetic adult males (8 individual samples/each group) by Trizol® (Invitrogen). The concentration of extracted RNA was quantified using NanoDrop. Quantitative Real-Time PCR (RT-qPCR) was performed using the LightCycler®480 Real-Time PCR system (Roche, Roche Applied Science, Mannheim, Germany) on cDNA samples. The collected RNA samples (1 µg) were subjected to reverse transcription using Superscript II (Fermentas, Lithuania). cDNA was diluted 20x and 4 µl were added to 6 µl of Syber®Green JumpStart™ Tag ReadyMix™ (Sigma) with primers (0.25 µmol). Following the reverse transcription (RT), quantitative real-time PCR (qPCR) was performed with the initial AmpliTaq activation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s, as described in [23]. The Hprt1 gene was selected as the best reference gene for our analyses from the panel of 12 control genes (TATAA Biocenter AB, Sweden). The relative expression of the target gene was calculated using the ΔΔCp method, based on qPCR efficiencies (E) and the crossing point (Cp).
difference (Δ) of an experimental sample (diabetic Wt, non-diabetic and diabetic Hif1a+/−) versus control-non-diabetic Wt (ratio = \((E_{target})^{\Delta C_p \ target(Mean \ control – Mean \ EXP)} / (E_{Hprt1})^{\Delta C_p \ Hprt1(Mean \ control – Mean \ EXP)}\) [25]. RT-qPCR data were analyzed using the GenEX5 program (http://www.multid.se/genex/). The differences in normalized Cp values were tested for statistical significance by one-way ANOVA followed by Dunnett’s multiple comparison post-test (all groups vs. non-diabetic Wt) with significance assigned at the P<0.05 level (Graph Pad, 2005). The primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/primer3/). Primer sequences are listed in Supplementary Table S1.

Morphological analysis

The adult hearts of diabetic and non-diabetic Wt and Hif1a+/− males were arrested in diastole by coronary perfusion with 5 mM cadmium chloride and 20 mM potassium chloride. After fixation with 4% paraformaldehyde overnight, the hearts were processed for paraffin histology. Adjacent sections (7 µm) were stained with Alcian Blue/Hematoxylin-Eosin (general histological staining), Picrosirius Red (collagen), TUNEL (apoptosis; #1684795, Roche), anti-collagen 1 (#203002, MD Biosciences), anti-smooth muscle actin (#A 2574, Sigma), anti-CD34 (blood vessels; #ab8158, Abcam), anti-VEGF-A (#sc-7269, Santa Cruz Biotechnology), and anti-connexin43/WGA (gap junctions and cell membranes; #C6219, Sigma/#W7024, Invitrogen). The nuclei were counterstained with Hoechst 33342 in fluorescence techniques or hematoxylin in dianaminobenzidine (DAB; #D3939, Sigma) visualization protocol. Myocyte size (minimum transverse diameter) was measured on sections stained with anti-CD34 visualized by DAB. The cardiomyocytes can be best approximated as rod-shape with an oval cross section. Any errors due to variation of the section plane are avoided by choosing the minor axis only in cells where the nucleus is present. Each analysis was repeated a minimum of 2 times on 2-3 individual samples per
genotype and included appropriate controls. The sections were analyzed under the Nikon Eclipse E400 fluorescent microscope or Leica SPE confocal microscope with a 40x magnification oil immersion objective, with NIS-elements or LCS program. VEGF-A\(^+\) areas were quantified using Image J software.

**Statistical analysis**

A one-way analysis of variance (ANOVA) was used to measure the statistical significance of differences among genotypes and experimental conditions. When a significant interaction was detected, the differences between subgroups were further compared using the post \(t\)-test (significance assigned at the \(P < 0.05\) level; Graph Pad, 2005; Graph Pad, San Diego, CA).

**Results**

**Echocardiographic evaluation of the LV function**

Five weeks after diabetes was established by repeated intraperitoneal streptozotocin injections, the body mass and LV mass gains of diabetic \(Hif1a^{+/−}\) and \(Wt\) males were lower compared to non-diabetic groups (Table 1). Diabetic females were less affected in the body and LV mass gain than diabetic males. Neither heart rate nor blood pressure differed among the groups, although the later parameter tended to increase in both \(Wt\) and \(Hif1a^{+/−}\) diabetic mice compared to the corresponding controls (males: \(P = 0.053\) and 0.066, respectively, Table 2). LV echocardiography did not reveal any difference between non-diabetic \(Wt\) and \(Hif1a^{+/−}\) mice. However, diabetes significantly influenced the LV echocardiographic parameters of \(Hif1a^{+/−}\) mice. The general trends of functional changes induced by diabetes were similar in males and females (Figure 1 and Table 2). LV fractional shortening (FS) was unaffected by genotype in non-diabetic \(Wt\) (males: 38.3 ± 0.6, females: 33.8 ± 1.3) and non-diabetic \(Hif1a^{+/−}\)
mice (males: 38.0 ± 0.9, females: 33.6 ± 0.6, Figure 1). A significant decrease in LV FS was detected in diabetic Hif1a+/− mice (males: 33.8 ± 1.2, females: 29.8 ± 1.0) but not in diabetic Wt animals (males: 36.9 ± 0.8, females: 32.8 ± 0.9, Figure 1). The differences in LV FS between non-diabetic and diabetic Hif1a+/− mice are also shown in representative M-mode echocardiographic recording (Figure 1B and C). Both diastolic and systolic AWT and PWT were significantly lower in diabetic Hif1a+/− males than in non-diabetic ones. Although, we observed similar tendencies in AWT and PWT parameters in diabetic Wt animals, these differences were not significant. Since male groups were more affected in LV echocardiographic parameters by diabetes than female groups, we only used males for our subsequent analyses.

**Cardiac gene expression profiling**

To explore the tissue specific molecular changes induced by diabetes, we analyzed the expression of 13 selected genes in the LV myocardium (Figure 2). We analyzed the expression of six HIF-1 target genes involved in glucose metabolism (Ldha; Slc2a1), autophagy (Bnip3l), insulin-like growth factor 2 (Igf2) and vasculogenesis (Vegfa; Flt1; Figure 2A). Under normal conditions, the HIF-1α heterozygous-null mutants showed a decreased cardiac transcription of three HIF-1 target genes, Vegfa, Igf2, and Ldha, reflecting Hif1a haploinsufficiency. The expression levels of mRNA of Vegfa were significantly affected by the combination of genotype and diabetes. The cardiac expression of Slc2a1, Flt1, and Bnip3l mRNA was significantly affected by diabetic conditions, but not by genotype. We also analyzed the expression of additional genes that are coding molecules involved in the regulation of signaling (Pdgfra; Tgfbr1; Cxadr; Itgav; Il6st; Ctss) and transcription (Gata2; Figure 2B). The expression levels of Cxadr, Pdgfra, and Il6st were increased in both Wt and
Hif1a+/− diabetic hearts compared to the non-diabetic Wt heart. The cardiac expression of Itgav was significantly decreased by the diabetic conditions in both Wt and Hif1a+/− groups. Interestingly, Gata2, Ctss, and Tgfbr1 mRNA levels were increased in the diabetic Hif1a+/−, but not in the diabetic Wt hearts, suggesting a combinatory effect of genotype and diabetes.

**Cellular and structural analysis**

To further investigate cellular changes associated with the diabetes-induced myocardial remodeling in the diabetes-exposed hearts, we analyzed the expression of collagen and the gap-junctional protein Cx43 (Figure 3A). The relative abundance of Cx43 was moderately decreased in the diabetic myocardium compared to non-diabetic groups in our study (Figure 3A). Cx43 protein levels were not different between groups or genotypes (Figure 3B and 3C). However, phosphorylated Cx43 at serine 368 (Ph-Cx43) was decreased in the diabetic Hif1a+/− mutant LV (Figure 3D). We also analyzed perivascular collagen 1(Col1) deposition in diabetic Hif1a+/− mutants compared to diabetic Wt and non-diabetic groups (Figure 3A). The protein levels of Col1 were significantly increased in the LV of diabetic Hif1a+/− group compared to other analyzed groups (Figure 3E). Quantitative measurements of myocyte width yielded identical values in all groups (data not shown). Additionally, we analyzed levels of apoptosis using TUNEL staining. We counted apoptotic cells in the LV, RV and septum. The number of apoptotic cells was moderately increased in the diabetic Wt hearts but not in the diabetic Hif1a+/− hearts, suggesting the genotype effect in cardiac responses to diabetic conditions (Figure 4). Thus, significant cardiac remodeling in the Hif1a+/− LV corresponds to the changes in the LV functional parameters in diabetic Hif1a+/− mutants compared to diabetic Wt and non-diabetic groups.
Since our RT-qPCR analysis demonstrated a significant combinatorial effect of genotype and diabetes on Vegfa mRNA expression, we analyzed the cardiac expression of VEGF-A, a key HIF-1 target gene product. VEGF-A is the essential modulator of neovascularization and diminished levels of VEGF-A have been associated with the impaired collateral vessel formation in the myocardial tissue of diabetic patients [17, 26]. We analyzed histological sections of Wt and Hif1a+/− diabetic and non-diabetic hearts to analyze the spatial expression of VEGF-A using immunohistochemistry (Figure 5A). The anti-VEGF staining was found to be limited to the wall of coronary vessels in all groups. The relative quantification of VEGF-A expression in the wall of coronary vessels showed decreased protein levels by 50% in non-diabetic Hif1a+/− compared to Wt, corresponding to the haploinsufficiency of Hif1a (Figure 5B). Furthermore, the VEGF-A protein levels were significantly decreased in the coronary vessels of diabetic Hif1a+/− and Wt hearts, indicating vascular changes in the diabetic heart.

**Discussion**

This study investigated the functional role of HIF1-pathways in cardiac responses to diabetic conditions, including changes in LV echocardiographic parameters, transcriptional profile modulations, and tissues remodeling. For the first time, we showed that HIF-1 regulated pathways are involved in the first manifestation of pathological changes induced by the diabetic environment in the heart. We showed that the globally decreased expression of Hif1a accelerated the early-phase pathological effects of diabetes on the heart. The echocardiographic parameters of the LV were significantly affected in diabetic Hif1a+/− animals. Impaired LV cardiac functions were accompanied by transcriptional changes and cardiac remodeling in the LV of diabetic Hif1a+/− mutants compared to diabetic Wt mice.
We used the streptozotocin model which has been proved to produce diabetes in animal models without systemic toxicity and characterized by hyperglycemia (blood glucose levels >13.9) and insulinopenia. Most studies using animal models with diabetes induced by streptozotocin revealed the decreased myocardial contractility and increased stiffness, resulting in both systolic and diastolic dysfunction at later stages of the disease (reviewed in [27, 28]). However, the onset of these changes, preceded by the altered gene expression, differs in individual studies and can be explained by differences in the severity of hyperglycemia, chronicity of diabetes, and experimental conditions. For example, both echocardiography and magnetic resonance imaging performed at the fourth week of diabetes in mice showed impaired indices of systolic and diastolic function [29], and diabetic rats at this stage of diabetes exhibited decreased maximal systolic elastance, indicating impaired intrinsic myocardial contractility [30]. However, Hoit et al. [31] observed the first signs of contractile dysfunction in rats only 5 weeks after streptozotocin injection and the overt systolic and diastolic dysfunction in 6 weeks. Consistent with this study, our experiments revealed only a minor decrease in relative LV wall thickness and unchanged fractional shortening in 5-week-diabetic Wt mice, indicating that heart function was still preserved at this stage. However, the harmful effects of diabetes were clearly more pronounced in Hif1a+/− mice as illustrated by the significantly decreased fractional shortening. It suggests that Hif1a+/− deficiency promotes the development of systolic dysfunction of the heart in association with diabetes. Our observations are in line with the increasing evidence that the HIF1-regulated pathways are compromised in the diabetic heart [14, 16, 17]. Studies in adult mice have demonstrated that HIF-1-dependent vascularization following femoral artery ligation or cutaneous wounding is impaired in diabetic mice, which can be rescued by experimental manipulations that increase HIF-1α expression [14, 15, 32].
The increased levels of Cxadr, Il6st, and Pdgfra in the LV of both Wt and Hif1a⁺/⁻ diabetic hearts correspond to the onset of pathological processes in diabetic cardiomyopathy. The overexpression of Cxadr, an adhesion molecule found at the intercalated disc of cardiomyocytes, produces cardiomyopathy in transgenic mice [33]. The transmembrane signal transduction protein gp130, encoded by Il6st, is a common receptor for the interleukin 6 family, which contributes to inflammatory processes, cardiac fibrosis and possibly to the development of type 1 and type 2 diabetes [34]. The activation of PDGFR-α induces collagen deposition, fibrosis, and inflammatory responses in an infarcted myocardium [35]. Observed increased levels of Slc2a1, the insulin-independent glucose transporter, indicate adaptation to the diabetic environment for better utilization of the glucose substrate [36].

The combinatory effect of the Hif1a⁺/⁻ genotype and diabetes was detected in the expression of Gata2, Cts, and Tgfbr1. The transcriptional factor GATA2 cooperates with HIF1-α and complements HIF-1 transcriptional regulation of pro-inflammatory genes in endothelial cells [37, 38]. Thus the increase of Gata2 mRNA in the diabetic Hif1a⁺/⁻ heart may indicate a compensation of reduced HIF-1α. Increased levels of Cts positively correlate with the extracellular matrix remodeling and inflammation [39]. Although the important regulatory role for HIF-1α in inflammation has been established [40], a cross-talk between CTSS and HIF-1 has not yet been observed. We observed an increased expression of Tgfbr1 mRNA in the LV of the Hif1a⁺/⁻ diabetic hearts, suggesting the activation of TGF-β signaling, which is associated with maladaptive changes in the composition of the extracellular matrix and fibrosis [41]. A cross-talk between TGF-β and HIF-1 pathways has been shown in the transcriptional regulation of Vefga, and Coll genes [42, 43].

Under normal conditions, apoptosis is a protective mechanism which eliminates old, useless, and damaged cells. Under diabetic conditions, increased apoptosis is associated with diabetes-related tissue damage in diabetic hearts [44]. Interestingly, we observed an increased
number of apoptotic cells in the diabetes-exposed Wt but not in the Hif1a<sup>+/−</sup> hearts. Since Wt mice were less affected by diabetes than Hif1a<sup>+/−</sup>, this increase in apoptosis in the early phase of diabetes-related tissue damage suggests a protective mechanism. The decreased sensitivity of Hif1a<sup>+/−</sup> cardiac tissue to apoptosis-induction signals may be a consequence of HIF-1α partial deficiency to induce apoptosis via p53, BNIP3, or/caspase-3 pathways.

Diabetic microvascular defects, associated with the increased incidence of chronic wounds and decreased post-ischemic vascularization, have been accompanied by a significant reduction of VEGF-A, a key HIF-1 target gene product [17, 26, 45]. The decreased levels of VEGF-A mRNA have been detected in ventricles from diabetic patients when compared to controls [26]. The decreased cardiac VEGF-A levels correlate with pathologically altered responses of diabetic patients to myocardial ischemia. In this study, we demonstrated the significantly decreased expression of Vegfa mRNA in diabetic Hif1a<sup>+/−</sup> compared to diabetic Wt mice. We observed discrepancies in the amplitude of mRNA and protein levels in the Hif1a<sup>+/−</sup> and Wt diabetic hearts. Although we cannot provide a definite explanation for these observations, these differences are likely due to the specific regulation of VEGF-A. VEGF-A is regulated at post-transcription, translation, and post-translation levels and both transcription and RNA stability can be enhanced by HIF-1α in response to normal as well as pathological conditions [46]. Our model provides the first proof that HIF-1α regulates Vegfa expression in the diabetic heart. The decreased levels of Vegfa in the Hif1a<sup>+/−</sup> diabetic heart correlated with the LV dysfunction and myocardial remodeling. Our results are indirectly supported by the study showing that the overexpression of Hif1a gene under the control of the myosin heavy chain promoter normalizes VEGF-A and HK-II levels and inhibits cardiac hypertrophy and fibrosis in the hearts exposed to diabetes for two months [18].
Conclusions

Our present data demonstrate that the combination of diabetes and \textit{Hif1a} haploinsufficiency accelerate the progression of diabetic cardiomyopathy, particularly a significant decrease in LV fractional shortening. This LV functional impairment has been accompanied by significant changes in the LV transcriptional profile, including \textit{Vegfa}, and myocardial remodeling. Our results highlight a critical link between diabetes, HIF-1\(\alpha\) regulation, and cardiovascular dysfunctions. Furthermore, clinical studies have demonstrated that polymorphisms at the HIF1A locus influence the presentation of ischemic heart disease and have been associated with type 2 diabetes [47, 48]. Taken together with the results presented here, these data raise the possibility that genetic variation at the HIF1A locus may also influence the increased risk for diabetic cardiomyopathy.

Abbreviations

\textit{HIF-1}\(\alpha\): hypoxia-inducible factor 1\(\alpha\); \textit{Slc2a1}: glucose transporter 1; \textit{Vegfa}: vascular endothelial growth factor A; \textit{Flt1}: Vegf receptor-1; \textit{Igf2}: insulin-like growth factor 2; \textit{Ldha}: lactate dehydrogenase A; \textit{Bnip3l}: BCL2/adenovirus E1B interacting protein 3-like; \textit{Cxadr}: coxsackie virus and adenovirus receptor; \textit{Il6st}: interleukin 6 signal transducer; \textit{Tgfbr1}: transforming growth factor beta receptor I; \textit{Itgav}: integrin alpha V; \textit{Pdgfra}: platelet derived growth factor receptor alpha; \textit{Gata2}: GATA binding protein 2; \textit{Ctss}: cathepsin S; \textit{Col1}: collagen 1; \textit{Cx43}: connexin 43.

Competing interests

The authors declare that they have no competing interests.
Authors’ contributions
RB conceived and designed experiments. FK designed and performed echocardiographic evaluations. DS designed and performed morphological and morphometric evaluations. LS prepared animals and processed tissues for morphological exams. FP and JN assisted in mice treatment and performed echocardiographic examinations. GP designed the study and wrote the paper. All authors contributed to the final discussion, read and approved the final manuscript.

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References


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<th>Genotype Parameter</th>
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<td>BW (g)</td>
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<td>104 ± 4</td>
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<td>RV/BW</td>
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<td>LV/BW</td>
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Values are means ± SEM from indicated number of animals in each group. Abbreviations:

BW, body weight; HM, heart mass; LV, left ventricular mass; RV, right ventricular mass.

Statistical significance was assessed by one-way ANOVA, *P<0.05 vs. non-diabetic Wt; ‡P<0.05 vs. non-diabetic Hif1a\(^{+/−}\).
Table 2 Mean systemic arterial blood pressure (MAP), heart rate (HR) and basal left ventricular echocardiographic parameters

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<th>Genotype</th>
<th>Parameter</th>
<th>Wt non-diabetic</th>
<th>Wt diabetic</th>
<th>Hif1a⁺⁻ non-diabetic</th>
<th>Hif1a⁺⁻ diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n)</td>
<td>MAP (mmHg)</td>
<td>83.0 ± 2.1</td>
<td>87.5 ± 1.5</td>
<td>84.1 ± 1.4</td>
<td>88.2 ± 1.6</td>
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<tr>
<td></td>
<td>HR (beats/min)</td>
<td>496 ± 23</td>
<td>471 ± 13</td>
<td>449 ± 40</td>
<td>440 ± 36</td>
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<td>LVD_D (mm)</td>
<td>3.60 ± 0.09</td>
<td>3.59 ± 0.04</td>
<td>3.47 ± 0.10</td>
<td>3.65 ± 0.06</td>
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<td>LVD_S (mm)</td>
<td>2.22 ± 0.07</td>
<td>2.27 ± 0.05</td>
<td>2.16 ± 0.08</td>
<td>2.42 ± 0.07</td>
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<td>AWT_D (mm)</td>
<td>0.84 ± 0.02</td>
<td>0.76 ± 0.01</td>
<td>0.89 ± 0.02</td>
<td>0.74 ± 0.03*</td>
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<td>PWT_D (mm)</td>
<td>0.84 ± 0.03</td>
<td>0.76 ± 0.01</td>
<td>0.92 ± 0.04</td>
<td>0.69 ± 0.03*</td>
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<td>AWT_S (mm)</td>
<td>1.30 ± 0.03</td>
<td>1.20 ± 0.04</td>
<td>1.27 ± 0.03</td>
<td>1.10 ± 0.05*</td>
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<td>PWT_S (mm)</td>
<td>1.21 ± 0.03</td>
<td>1.09 ± 0.03</td>
<td>1.20 ± 0.04</td>
<td>1.00 ± 0.04*</td>
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<td>Female (n)</td>
<td>MAP (mmHg)</td>
<td>84.6 ± 2.1</td>
<td>88.5 ± 2.2</td>
<td>84.8 ± 0.04</td>
<td>88.1 ± 1.3</td>
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<td>HR (beats/min)</td>
<td>504 ± 7</td>
<td>478 ± 15</td>
<td>484 ± 16</td>
<td>449 ± 17</td>
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<td>LVD_D (mm)</td>
<td>3.52 ± 0.08</td>
<td>3.52 ± 0.06</td>
<td>3.45 ± 0.04</td>
<td>3.46 ± 0.04</td>
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<td>LVD_S (mm)</td>
<td>2.33 ± 0.07</td>
<td>2.37 ± 0.06</td>
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<td>2.43 ± 0.06</td>
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<td>AWT_D (mm)</td>
<td>0.75 ± 0.02</td>
<td>0.75 ± 0.01</td>
<td>0.72 ± 0.02</td>
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<td>PWT_D (mm)</td>
<td>0.79 ± 0.02</td>
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<td>AWT_S (mm)</td>
<td>1.12 ± 0.04</td>
<td>1.05 ± 0.02</td>
<td>1.06 ± 0.01</td>
<td>0.98 ± 0.02*</td>
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<td>PWT_S (mm)</td>
<td>1.10 ± 0.02</td>
<td>1.05 ± 0.02</td>
<td>1.09 ± 0.02</td>
<td>0.99 ± 0.02*</td>
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</tbody>
</table>

Values are mean ± SEM. LVD_D - diastolic cavity diameter, LVD_S - systolic cavity diameter, AWT_D - diastolic anterior wall thickness, PWT_D - diastolic posterior wall thickness, AWT_S - systolic anterior wall thickness, PWT_S - systolic posterior wall thickness. Statistical significance was assessed by one-way ANOVA, *P<0.05 vs. non-diabetic Wt.
Figure 1 Echocardiographic assessment of LV systolic function in Wt and Hif1a+/− mice. 

(A) The exposure to the diabetic environment resulted in a significant decrease of fractional shortening (FS) in Hif1a+/− mice but not in Wt animals. The values are mean ± SEM, *P<0.05, Wt and Hif1a+/− non-diabetics vs. diabetic Hif1a+/−. Representative M-mode recordings of left ventricular structures in long axes view in a non-diabetic (B) and diabetic (C) Hif1a+/− mouse with FS = 38.7 and FS = 32.8, respectively.

Figure 2 Gene expression changes in the LV of Wt and Hif1a+/− diabetic mice. The expression of genes was analyzed using RT-qPCR: (A) direct HIF-1α target genes and (B) genes coding signaling molecules, growth factors, cytokines, and transcription factors. The relative expression levels were quantified using the ∆∆CT method. The data represent the expression of mRNA relative to the non-diabetic Wt expression of mRNA, normalized by the housekeeping mRNA of Hprt1. The values are means ± SEM (each experiment in duplicate; n = 8 per group of diabetic Wt and Hif1a+/−, non-diabetic Hif1a+/−; n = 7 per non-diabetic Wt), *P<0.05. Abbreviations: glucose transporter 1 (Slc2a1), vascular endothelial growth factor A (Vegfa), Vegf receptor-1 (Flt1), insulin-like growth factor 2 (Igf2), lactate dehydrogenase A (Ldha), BCL2/adenovirus E1B interacting protein 3-like (Bnip3l), coxsackie virus and adenovirus receptor (Cxadr), interleukin 6 signal transducer (Il6st), transforming growth factor beta receptor I (Tgfbr1), integrin alpha V (Itgav), platelet derived growth factor receptor alpha (Pdgrfa), GATA binding protein 2 (Gata2), cathepsin S (Ctss)

Figure 3 Effects of diabetes on structural remodeling and protein levels in the Wt and Hif1a+/− LV. (A) Representative of immunofluorescence confocal and light microscopy images of the hearts stained with anti-connexin 43 (Cx43, red) together with fluorescein-labeled wheat germ agglutinin (WGA, green); with picrosirius red (PSR, polarizing microscopy); and
with anti-collagen 1 (Col1, red), autofluorescence (auto). The nuclei were counterstained with Hoechst 33342 (blue). Confocal images are stacked Z-plane sections from confocal microscopy. Scale bar: Cx43/WGA 25 µm, PSR 50 µm, Coll1 25 µm. (B) Representative Western blot analysis of protein levels in the isolated LV of Wt and Hif1a+/− hearts, and (C-E) relative quantification of protein levels of Cx43, phosphoCx43 (pCx43), and Col1 normalized to the loading control ATP5a (n = 3 per group). *Significant effect of genotype and diabetes, P<0.05.

Figure 4 Apoptosis in the diabetic and non-diabetic hearts of Wt and Hif1a+/− mice. The apoptotic cells were detected with TUNEL assay. The apoptotic cells were counted in the whole heart, including the myocardium of left and right ventricles, and the atrioventricular septum. The values represent means ± SEM (n = 3 individuals x 3 heart sections per group). *P<0.05, non-diabetic Wt vs. diabetic Wt; diabetic Wt vs. diabetic Hif1a+/−.

Figure 5 Diabetes-induced changes in cardiac VEGF-A expression. (A) Confocal imaging of transverse sections of Wt and Hif1a+/− hearts stained with anti-VEGF-A antibody (green) showed VEGF-A expression in coronary blood vessels (white arrow). Hoechst 33342 (blue) was used as a nuclear counterstain. Images are stacked Z-plane sections from confocal microscopy. Scale bar: 10 µm. (B) Relative quantification of VEGF-A expression in the blood vessel wall. The quantification of VEGF-A+ area was determined as a ratio of VEGF-A+ area per total vessel area in the field of view using ImageJ. Data are presented as the mean ± SEM (n = 4 - 8 vessels x 2 individuals per group). †P<0.01, non-diabetic Wt vs. non-diabetic Hif1a+/−; *P<0.001, non-diabetic Wt vs. diabetic Wt or diabetic Hif1a+/−.
Figure 1

A

- Wt
- Hif1a+/

Males

- non-DIA
- DIA

Females

B

C

m+ndh-922
FS 38.7

m+dh-943
FS 32.8

m+ndh-922
FS 38.7

m+dh-943
FS 32.8
Figure 4

The bar graph shows the number of apoptotic cells in the heart for two groups: Wt and Hif1a+/-, under non-DIA and DIA conditions. The graph indicates a significant difference between the two conditions, with DIA leading to a higher number of apoptotic cells, as indicated by the asterisks.
Additional files provided with this submission:

Additional file 1: Table S1primers.docx, 16K
http://www.cardiab.com/imedia/8515459031103011/supp1.docx