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The Effect of Hypoxia on Irisin Expression in HL-1 Cardiomyocytes

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Abstract. *Background/Aim: Cardiovascular diseases (CVD) are the leading cause of death worldwide. In 2019, 523 million people were diagnosed with CVD, with 18.6 million deaths. Improved treatment and diagnostics could reduce CVD's impact. Irisin (Ir) is crucial for heart function and may be a biomarker for heart attack. Ir is a glycoprotein with sugar residues attached to its protein structure. This glycosylation affects Ir stability, solubility, and receptor interactions on target cells. Its secondary structure includes a fibronectin type III domain, essential for its biological functions. Ir helps cardiomyocytes to respond to hypoxia and protects mitochondria. The aim of the study was to determine the FNDC5 gene expression level and the Ir level in HL-1 cardiomyocytes subjected to hypoxia. Materials and Methods: We examined the effect of hypoxia on the expression levels of the FNDC5 gene and those of Ir in mouse cardiomyocytes of the HL-1 cell line. Real-time PCR (RT-PCR) was used to estimate the expression levels of the FNDC5 gene. Western blot and immunofluorescence methods were used to analyze the Ir protein levels. Results: Analyses showed an increased Ir level in HL-1 cardiomyocytes in response to hypoxia. This is the first study to confirm the presence of Ir in HL-1 cells. Conclusion: The observed increase in Ir expression in murine cardiomyocytes is associated with the hypoxic environment and can be potentially used to diagnose hypoxia and CVD.*

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Cardiovascular diseases (CVD) are the leading cause of death worldwide, with myocardial infarction (MI) being the most common reason (1). In 2019, 523 million patients were diagnosed with CVD, resulting in 18.6 million deaths (2). Recent data do not reliably reflect the real incidence of CVD due to the decrease in hospitalizations during the COVID-19 virus epidemic (3). In developed countries, a significant decline in CVD incidence has been observed over the past three decades, which has been attributed to improved access to treatment and faster diagnosis (4, 5, 6). Several biomarkers are used to diagnose CVD. Irisin (Ir) shows diagnostic potential for cancer (7, 8) and recent reports suggest it may also emerge as a new biomarker for MI (9, 10).

Ir is a protein produced by muscle cells during exercise that converts white fat tissue into brown fat, increasing energy expenditure and improving metabolism (11). Ir consists of 112 aa residues. Ir is produced by cleavage from the prohormone FNDC5, encoded by the *FNDC5* gene (12). Once produced, Ir is cleaved from FNDC5 and released into the bloodstream where it can exert its effects on various tissues. The FNDC5 prohormone consists of a signal peptide, a fibronectin type III-like domain and a hydrophobic C-terminal domain (13). Ir is released after proteolytic cleavage of the extracellular portion of the FNDC5 protein containing the fibronectin type III domain (14). Chemically, Ir is a glycoprotein, which means it contains sugar residues attached to the protein. Glycosylation of Ir can affect its stability, solubility and interactions with receptors on the surface of target cells. Ir has a secondary structure typical of proteins containing a fibronectin type III domain, which is critical for its biological functions (15).

Expression of the *FNDC5* gene is regulated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (16) and estrogen-related receptor alpha (ERRα), which are associated with each other (17). PGC-1α is a transcriptional coactivator involved in the regulation of FNDC5 expression in various tissues (18). In 2012, Boström *et al.* described the release of Ir from skeletal muscle fibres

into the serum (16). Subsequent studies have shown that the highest levels of Ir among normal cells are observed in cardiomyocytes (19). In cardiomyocytes, Ir is released from FNDC5 by one of the proteases of the ADAM family, most likely the ADAM10 protease (20).

Ir stimulates cardiomyocyte proliferation and angiogenesis (21) and helps reduce the effects of myocardial ischaemia by improving mitochondrial function (22) and protecting it from damage (23). Mitochondria in cardiomyocytes undergo degenerative changes under the influence of oxidative stress (24). Abnormal mitochondrial structure and function, and thus disturbances in their metabolism, play a crucial role in cellular stress and apoptosis (25). Ir also influences the modulation of mitochondrial function (26). The potential protective effect of Ir on cardiomyocytes in hypoxia is attributed to the improvement of mitochondrial function, reduction of autophagy and decrease in apoptosis 27,28). These findings are supported by *in vitro* studies showing that Ir administration to cells restores the integrity of mitochondrial structures. Inhibition of pore opening and reduction of mitochondrial swelling were observed, improving mitochondrial respiratory function (29, 30). This was confirmed by the research of Moscoso *et al*. (31) who observed that rat cardiac myoblasts of the H9C2 cell line subjected to hypoxia, showed greater survival after administration of Ir. However, Xie *et al*. (32) observed increased metabolism, inhibition of proliferation and enhanced differentiation of H9C2 cells after Ir administration. It is unclear whether Ir secretion is a side effect of cardiomyocyte damage or a deliberately secreted molecule reflecting cardiac perfusion and influencing its functional potential (33).

Studies on rats have shown that the level of Ir in MI was decreased in the serum and increased in the cardiomyocytes (34). During MI hypoxia occurs, followed by cardiomyocyte necrosis. Hypoxia can increase level of hypoxia-inducible factor-1α (HIF-1α), a protein that serves as a marker of hypoxia. HIF-1α is synthesised by cells continuously. Under hypoxic conditions, HIF-1α translocates to the nucleus and dimerises with HIF-1β. The dimer binds to hypoxia response elements (HREs) in the promoters of genes that respond to reduced oxygen levels (35). The transcription factor HIF-1α plays a crucial role in the cellular response to systemic oxygen levels. Under normoxic conditions, the VHL-dependent proteolytic pathway rapidly degrades HIF1A. However, under hypoxic conditions, the degradation of HIF1A is halted, causing its levels to accumulate. As a result, HIF1A can bind to HIF1B, allowing them to jointly exert transcriptional functions on target genes. The presence of HIF-1α in cells is therefore associated with the pathophysiology of vascularisation, angiogenesis and their energy metabolism (36).

The aim of the study was to determine the expression level of the *FNDC5* gene and the level of Ir in cardiomyocytes of the HL-1 line subjected to hypoxia.

Materials and Methods

Cell culture. Murine cardiac myocyte cell line (HL-1) (37) was cultured in Claycomb's medium (Sigma-Aldrich, St. Louis, MO, USA) with the addition of 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% L-glutamine with streptomycin and penicillin solution (Sigma-Aldrich) and 0.1 mM norepinephrine solution (Sigma-Aldrich) in ascorbic acid (Chempur, Piekary Slaskie, Poland). Cell cultures were incubated in a HeraCell 150i incubator (ThermoFisher Scientific, Wilmington, DE, USA) at 37˚C in a 5% CO_2 atmosphere and 95% humidity. The confluence of the cell culture did not exceed 70% and passaging was provided with TrypLe (Gibco, ThermoFisher Scientific). Additionally, cell culture was maintained for 24 hours under hypoxic conditions (1% O_2) in a hypoxic live-cell imaging confocal microscope chamber (1% O_2) (Fluoview FV3000 confocal microscope, Olympus, Tokyo, Japan). The experiment was repeated. Three biological replicates and three technical replicates were performed.

Immunofluorescence. For 24 h microculture, 600 μl of 2×10^4 cells per well were set up on slides with Millicell EZ 8-well glass slides (Merck, Darmstadt, Germany) and incubated at 37˚C for 24 h. Subsequently, microcultures were transferred to a live-cell imaging confocal microscope chamber (Fluoview FV3000 confocal microscope). The cultures were maintained for 24 h under hypoxic conditions (1% O_2) as well as under normoxic conditions. After the incubation, the slides were fixed using 4% formaldehyde. Then, the cells were incubated with the primary antibody: polyclonal rabbit anti-irisin/FNDC5 (dilution 1:50; Cat. No. NBP2-14024; Novus Biologicals, Centennial, CO, USA) and Rabbit monoclonal anti-HIF-1a (clone D1S7W, Cat. No. 36169; RRID: AB_2799095, Cell Signaling, Danvers, MA, USA) at 4˚C overnight. Next, the slides were incubated for 1 h at room temperature (RT) with donkey anti-rabbit secondary Alexa Fluor 568 conjugated antibody (1:2,000 dilution; clone, Cat. No. ab175470; Abcam, Carlsbad, CA, USA) and were mounted using the ProLong Gold Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA). The observations were made at objective 60×/1.40 oil using Fluoview FV3000 confocal microscop (Olympus) coupled with Cell Sense software (Olympus). Three biological replicates and three technical replicates were performed.

Western blotting. Protein expression by western blot analysis was performed using the HL-1 cell line cultured under hypoxic and normoxic conditions. For each analysis, $5\text{-}6\times10^6$ HL-1 cells in the exponential growth phase were taken. After washing with ice-cold phosphate-buffered saline (PBS), cells were lysed with RIPA buffer [50 mM Tris HCl; 150 mM NaCl; 0.1% SDS; 1% Igepal (CA-630,); 0.5% sodium deoxycholate; protease inhibitor cocktail (Merck); 0.5 mM PMSF] for 20 min on ice. The concentration of isolated cell lysate protein was measured using the Pierce BCA Protein Assay Kit (Thermo-Fisher, Waltham, MA, USA) and a NanoDrop 1000 spectrophotometer (Thermo-Fisher, Waltham, MA, USA). Protein was denatured at 95˚C for 10 min in sample loading buffer GLB (250 mM Tris-HCl, 40% glycerol, 20% β-mercaptoethanol, 8% SDS and bromophenol blue), transferred to PVDF membrane (Millipore, Burlington, MA, USA) and blocked with 2% non-fat milk (Bio-Rad, Marnes-la-Coquette, France) in 0.1% TBST [Tris-buffered saline (TBS) with Tween 20] for 1 h at RT. Ir expression was detected using a rabbit polyclonal anti-irisin/FNDC5 antibody (dilution 1:200 in

0.5% milk in 0.1%TBST; Cat. No. NBP2-14024; Novus Biologicals). Then, the membrane was incubated with rabbit polyclonal anti-irisin/FNDC5 antibody (dilution 1:200; Cat. No. NBP2-14024, Novus Biologicals) overnight at 4˚C. Subsequently, the membrane was incubated with the secondary horseradish peroxidase conjugated with donkey anti-rabbit antibody diluted in 0.5% milk in 0.1% TBST (dilution 1:3,000; Cat. No. 711-035-052; Jackson ImmunoResearch, Cambridgeshire, UK) for 1 h at RT. The proteins were visualized using the Luminata Classico Western HRP Substrate (Millipore). The membrane was stripped and incubated with monoclonal mouse anti-actin antibody (dilution 1:500, clone AC-40, Cat. No. A4700, Merck) used as the loading control. The data were documented for exposure times ranging from 2 sec to 30 min in the Chemi-Doc XRS Molecular Imager apparatus (Bio-Rad). The optical density of the protein band was measured with the use of the Image Lab (Bio-Rad) software. The experiment was repeated. Three biological replicates were performed and three technical replicates for each of them were performed.

Real-time PCR (RT-PCR). RT-PCR was performed for HL-1 cells cultured 24 h under hypoxic and normoxic conditions. RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA). Reverse transcription reactions were performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Waltham, MA, USA). The expression level of *FNDC5* (FNDC5; TaqMan Gene Expression Assay Applied Biosystems; Mn01181543_1) was assessed using the 7900HT Fast Real-Time PCR System (Applied Biosystems). Thermal cycling conditions were as follows: polymerase activation at 50˚C for 2 min, preliminary denaturation at 94˚C for 10 min, denaturation at 94˚C for 15 sec, annealing of primers and probes, and synthesis at 60˚C for 1 min, for 40 cycles. Relative expression (RQ) of FNDC5 mRNA was calculated using ΔΔCt method. Analysis was performed using RQ Manager 1.2 software (Applied Biosystems). Results were normalised to the reference gene β-actin (ACTB; TaqMan Gene Expression Assay, Applied Biosystems; Mm00607939_s1). The evaluation of *FNDC5* gene expression by real-time PCR was repeated. Two biological replicates were performed. Three technical replicates were performed for each of them.

Statistical analysis. Kruskal-Wallis and Mann-Whitney tests were used to compare the groups of data that did not meet the assumptions of the parametric test. The statistical analysis was made using Prism 5.0 (GraphPad, La Jolla, CA, USA). The results were considered statistically significant at $p<0.05$.

Results

Immunofluorescence (IF). IF analysis revealed the expression of Ir in the cytoplasm of murine HL-1 cardiomyocytes. Cells exposed to hypoxia showed a significantly higher level of cytoplasmic Ir expression (mean value 787.12±39.63 SD) compared to cells cultured under normoxic conditions (mean value 76.54±5.09 SD; Mann-Whitney *U*-test; $p<0.0001$) (Figure 1).

Under normoxic conditions the Hif1α factor was detected in the cytoplasm, whereas under hypoxic conditions it was detected in the nuclei of cardiomyocytes. The localization of Hif1α in the nuclei of cardiomyocytes confirmed the hypoxic conditions of the experiment. The IF method was used to localize the expression of Ir and Hif1α in cardiomyocytes. The IF expression results of both proteins are shown in Figure 2.

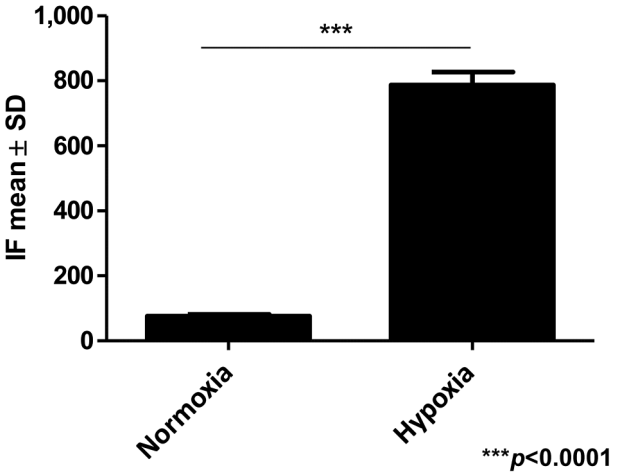

Figure 1. *Comparison of the Ir expression levels by immunofluorescence in mouse cardiomyocytes of HL-1 cells line cultured in normoxic and hypoxic conditions ($p<0.0001$). Ir: Irisin.*

Western blotting. Western blot analysis was performed to compare the expression of Ir in HL-1 cardiomyocytes cultured in normoxic and hypoxic conditions. Densitometric analysis revealed a significantly higher level of Ir in cells exposed to hypoxia ($p=0.0308$) (Figure 3).

RT-PCR. RT-PCR method analysis showed no statistically significant differences in *FNDC5* expression levels. However, an upward trend was observed in cells exposed to hypoxia (Figure 4).

Discussion

A biomarker for CVD should fulfill several important criteria. It should detect the disease at its early stage and be specific to a particular disease entity. Moreover, this biomarker should have a high prognostic ability to predict the progression of the disease and support physicians in choosing a proper therapeutic strategy. Currently, the research for new biomarkers to improve diagnostics is still ongoing (38). For CVD, the best diagnostic markers are cTnT and cTnI troponins. They are considered effective biomarkers because troponins are detected by intermediate sensitivity immunoassays. The introduction of high-sensitivity immunoassays into diagnostics allowed to determine lower concentrations of troponin molecules, which were previously not detected by the moderately sensitive immunoassays (39). This is crucial in detecting early stages of CVD (40). Markers, such as CPK-MB, GPBB and myoglobin

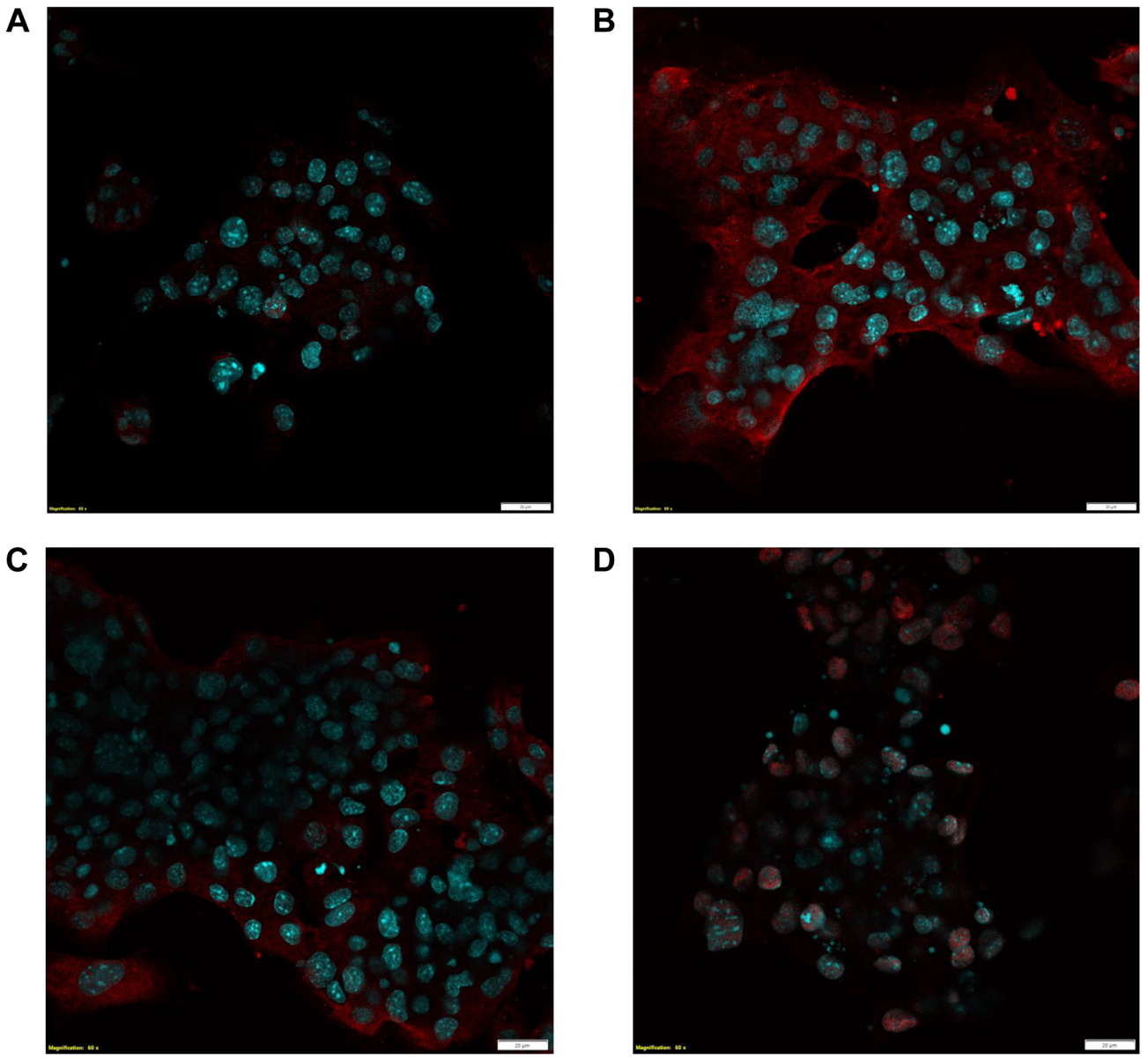

Figure 2. *Comparison of Ir and Hif1α expression by confocal microscopy in the HL-1 cells subjected to hypoxia and normoxia. (A) Normoxia - low expression of Ir, magnification 60×. (B) Hypoxia - high expression of Ir, magnification 60×. (C) Normoxia - absence of nuclear expression of Hif1α, magnification 60×. (D) Hypoxia - nuclear expression of Hif1α, magnification 60×. Ir: Irisin.*

are highly sensitive but have low specificity in diagnosing of CVD, and it is, therefore, believed that they cannot be used as primary CVD biomarkers (41).

The discovery of new diagnostic methods for ischemic diseases such as MI seems to be highly significant and may support the process of diagnosis and treatment. Currently, an intensive research is carried out regarding the use of adipokines as potential new CVD biomarkers (42). Ir could be one of these potential biomarkers. Our study in an *in vitro* model provides information on changes in the Ir levels occurring in cardiomyocytes of the HL-1 cell line in hypoxia. To our knowledge, the levels of Ir in HL-1 cardiomyocytes have not been studied so far. White *et al*. (43) revealed that HL-1 cells are an ideal *in vitro* model for studying the impact of pathological conditions, such as hypoxia and hyperglycemia, on cardiac function. Analysis of gene expression in HL-1 cells indicates that they have a similar gene expression profile to adult cardiomyocytes (37). Moreover, HL-1 cells show an analogous response to ischemia and reperfusion as Wistar rat cardiomyocytes (44).

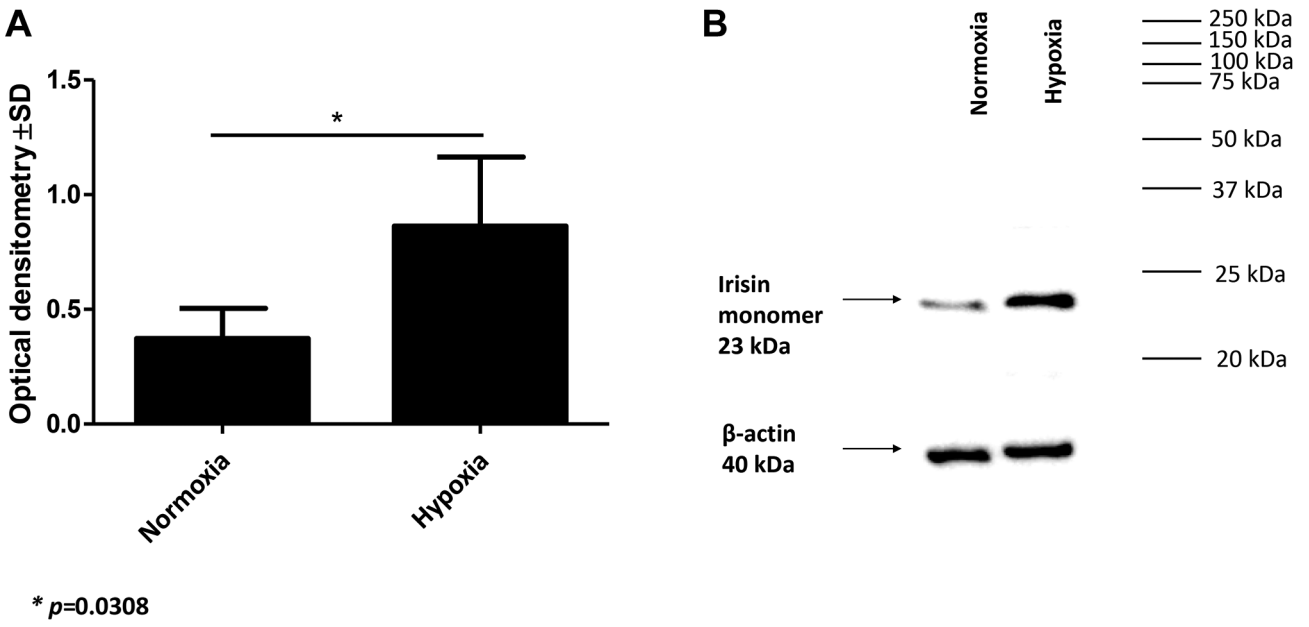

Figure 3. *Comparison of Ir expression by western blotting in the HL-1 cells subjected to hypoxia and normoxia (A). Densitometric analysis of Ir protein levels (B). Ir: Irisin.*

In our study, we observed an increase in Ir levels in cells cultured under hypoxic conditions. Contrary to us, Moscoso *et al*. (31) detected a decrease in Ir levels probably because a different cell line was used. The Hypoxic condition was achieved using Baker Ruskinn's InvivO2 200 Bridgwater chamber, at a lower oxygen concentration (0.1% O_2, 5% CO_2 and N_2 balance) than the 24-h cellular oxygen requirement, to mimic the pathophysiological ischemic environment. They also observed greater survival of cardiomyocytes when the cells were previously provided with Ir. Kuznetsov *et al*. (45) revealed that the level of PGC-1α in HL-1 cells is higher than the levels of this protein in H9C2 cells. This difference may be important in explaining the discrepancy in observed Ir levels in the hypoxia model between the HL-1 and H9C2 cardiomyocyte lines. The Ir examined in our studies is encoded by the *FNDC5* gene, the expression of which is regulated by the coactivator gamma and activated by proliferator-1α (PGC-1α) (16). PGC-1α is a transcription factor that regulates the expression levels of genes encoding the FNDC5 protein in different tissue types (11). The altered levels of PGC-1α in the H9C2 lines studied by Moscoso *et al*. and the HL-1 line studied by our team may explain the discrepancy in results and the observed increase in Ir expression. Our cell line, due to its higher expression of PGC-1α, is characterised by a higher expression of FNDC5 and Ir.

Studies by Aydin *et al*. (34) showed that the amount of Ir in the serum was decreased after MI. The researchers revealed that serum Ir levels decreased within 6 to 48 h of an acute MI event. Only 72 h after MI, Ir level gradually returned to pre-MI level.

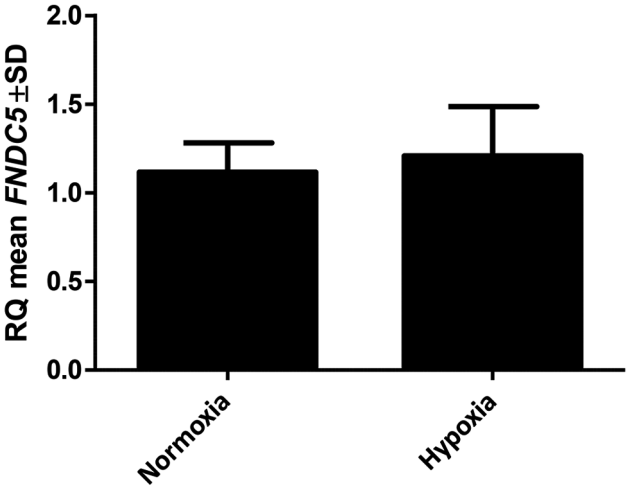

Figure 4. *Comparison of FNDC5 mRNA expression with use of the real-time PCR method.*

The decrease in Ir level in serum may indicate its accumulation in cardiomyocytes during hypoxia (46) and it may suggest a protective effect of Ir. Fan *et al*. (23) observed the effect of Ir on the activation of the AMPK pathway in a study of H9C2 cells subjected to 30 min of hypoxia and 4 h of reoxygenation with simultaneous high glucose levels. Providing medium with human Ir maintained the proper functioning of mitochondria through the AMPK kinase pathway and was associated with an increase in cardiomyocytes survival. Ischaemia stimulates

increased transcription and protein levels of Ir in the myocardium. Ir may protect the heart from ischaemia and reperfusion injury by supporting mitochondrial function (47). In addition, high levels of Ir reduce the expression of apoptotic proteins, including active caspase-3, PARP and annexin V (47). On the other hand, Yue *et al*. (48) found that early administration of Ir to the medium containing mouse cardiomyocytes may also lead to a reduction in ischaemia-reperfusion damage, promotion of survival, reduced cell apoptosis and decreased caspase 3 activity in these cells.

This may suggest the potential use of Ir in the therapy of patients with reperfusion injury. However, further studies are needed to explain the protective effect of Ir on cardiomyocytes. During MI, there is a noticeable increase in energy demand due to the reduced production of ATP derived from cellular respiration. Due to the limited availability of oxygen, ATP level in the myocardium decreases by half within 30 min after MI. The ATP stored in the heart is sufficient to maintain cellular homeostasis for a short period after MI. Studies by Liao *et al*. (49) confirmed the cardioprotective effect of Ir in the mice model. The researchers revealed that the administration of Ir, injected intraperitoneally after MI, led to increased angiogenesis in the heart muscles and reduced necrosis of cardiomyocytes, while having no effect on their proliferation. Similarly, Yue *et al*. (48) suggested that Ir may effectively protect cardiomyocytes from ischaemia-induced damage by reducing endoplasmic reticulum (ER) stress-induced apoptosis. Their experiments were performed on primary neonatal mouse cardiomyocyte lines isolated from C57BL/6 mice. Meanwhile, Sundarrajan *et al*. (50) administered human Ir intraperitoneally to zebrafish (Danio rerio). They observed the effect of Ir on increasing cardiac stroke volume, heart rate and cardiac output. Ir may not only prove to be a potential biomarker for CVD, but also a therapeutic implement due to its positive effects on cardiomyocyte survival and function.

The present study is the first to confirm the presence of Ir in the cytoplasm of mouse HL-1 cardiomyocytes and to detect an increase in its levels during 24 h of hypoxia. Ir seems to be a potential new diagnostic marker in CVD. The increase in Ir levels in mouse cardiomyocytes after hypoxia may suggest the potential usefulness of this protein as a marker in the diagnosis of CVD . In order to verify the Ir usefulness of Ir level assessment in the diagnosis of CVD and MI, further studies should be carried out analyzing the Ir level after hypoxia in human cardiomyocytes. Additionally, further studies are required to investigate the significance of Ir level increase during hypoxia, as well as the mechanism of Ir secretion and the factors influencing these processes.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study

Authors' Contributions

Conceptualization: MG, KN and PD; Methodology: MM, AK, AR, KJ and KN; Resources: AR, MM and PD; Data curation: KJ, KN, and AK; Investigation: MG, MM and KN; Project administration: MG, PD and KN; Formal analysis: KN; Supervision: PD; Validation: KN and PD ; Visualization: AK; Writing - original draft: MG, UC and KN; Writing - review & editing: PD, UC and KN.

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