The Pivotal Role of miRNA-21 in Myocardial Metabolic Flexibility in Response to Short- and Long-term High Glucose Treatment: Evidence in Human Cardiomyocyte Cell Line

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Abstract

Background

miRNA-21 is a micro-RNA widely studied for its implications in several biological functions, including apoptosis, inflammation, fibrosis, and metabolism. Interestingly, miRNA-21 is a crucial regulator of developing cardiac diseases, but its role is controversial, and it is necessary to clarify its function in pathophysiological events of diabetic cardiomyopathy.

In the present study we clarified the protective role of miRNA-21 at cardiac level and evaluated the involvement of miRNA-21 in high glucose induced-acute and chronic cardiac damage.

Methods

Human ventricular cardiac myoblasts AC16, treated and not with miR-21 inhibitor, were exposed to high glucose (33 mM) for 2 and 7 days, and the expression of fibrosis, inflammation, apoptosis, oxidative stress markers were assessed using western blotting. Further, cardiac energetic metabolism was evaluated by measuring both the expression of glucose transporters and regulators of lipids using western blotting analysis and key mitochondrial function parameters (oxygen consumption rate and proton production rate) using Seahorse technology.

Results

Short-term high glucose treatment induced a significant increase in miR-21 expression ($p < 0.05$) that was associated with an increase in hydrogen ion flux and energy potential dissipation without any change in energy production or increase in the expression of genes involved in cellular damage. The reduction of miR-21 expression levels ($p < 0.05$), observed after long-term (7 days) high glucose treatment, induced the activation of inflammation, apoptosis pathways and compromises mitochondrial function as demonstrated by the incapacity to answer energy demand and the impairment of physiological mitochondrial function ($p < 0.05$).

Conclusion

In human cardiomyocytes, the abundance of miR-21 takes part in the first defense mechanism against cardiac insult and its cardioprotective effect depends on the time of exposure to the injury. Moreover, miRNA-21 regulates mitochondrial respiration and the ability of cells to select the most appropriate substrate for ATP production in a given environment.

Background

MicroRNAs (miRNAs) are non-coding RNAs that negatively regulate the expression of their target genes and various cellular activities (1). Since they are involved in many diseases, their interest has dramatically increased during recent years (2). MicroRNA-21 is widely studied for its implication in several biological

functions, including apoptosis, inflammation, fibrosis, and metabolism (3, 4). In addition, miRNA-21 antagonizes cell apoptosis by Phosphatase and Tensin Homolog (PTEN), downregulates Programmed Cell Death 4 (PDCD4) and apoptotic markers such as BCL-2-like protein 4 (BAX) and caspase-3 (5–7). Furthermore, it exerts anti-inflammatory activity by reducing the rate of tumor necrosis factor (TNF)-alpha and inhibiting TLR4/NF-kB pathway with the consequent block of pro-inflammatory factors release (6). Moreover, miRNA-21 regulates fibrosis mechanisms, controlling the secretion of growth factors and fibroblast survival through TGF-beta/Smads, PI3K/AKT, and ERK/MAPK mitogen-activated kinase pathways (4, 8).

Interestingly, miRNA-21 also participates in cardiac glucose and lipid metabolism by promoting glucose uptake and glycolysis and down-regulating genes involved in fatty acid oxidation, such as cardiac PPAR- α (9). Recent studies also demonstrated the role of miRNA-21 in the progression of diabetic cardiomyopathy (DC) (8), characterized by the co-existence of adverse structural remodeling, including cardiac fibrosis and hypertrophy, and early-onset diastolic dysfunction and late-onset systolic dysfunction (10). Several molecular and cellular mechanisms such as metabolic disturbances, insulin resistance, formation and crosslinking of advanced glycation end products, mitochondrial damage, oxidative stress, inflammation, and cell death (10) have been identified for explaining DC. Intriguingly, high-glucose treatment related upregulation of miRNA-21 displayed a protective effect of cardiomyocytes toward apoptosis (11). In addition, miRNA-21 protected against cardiomyopathy-induced diastolic dysfunction by decreasing reactive oxygen species (ROS), elevated nitric oxide (NO), and improved cardiac hypertrophy in db/db mice (12). Notwithstanding, a negative impact of miRNA-21 on DC development has also been shown. In particular, the up-regulation of miRNA-21 resulted in myocardial fibrosis-inducing the transformation of cardiac fibroblasts to myofibroblasts, inducing the change with consequent myocardial fibrosis with further damage to the heart (13).

Therefore, miRNA-21 is a crucial regulator of developing cardiac diseases, but its role is controversial, and it is necessary to clarify its function in pathophysiological events of diabetic cardiomyopathy.

In the present study we clarified the protective role of miRNA-21 at cardiac level and evaluated the involvement of miRNA-21 in high glucose induced-acute and chronic cardiac damage.

Specifically, human ventricular cardiac myoblasts AC16, treated and not with miRNA-21 inhibitor, were exposed to high glucose for 2 and 7 days, and fibrosis, inflammation, apoptosis, and oxidative stress markers were evaluated. In addition, energetic metabolism, as variations of key mitochondrial function parameters, was also assessed using Seahorse XF.

Methods

Cell cultured

The AC16 human cardiomyocyte cell line was purchased from EMD Millipore (Darmstadt, Germany cat# SCC109). The cell line was tested and authenticated following the manufacturer's instruction, and it was

negative for mycoplasma contamination. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) / F12 (Microgem cat# AL215A) containing 10% fetal bovine serum (FBS, Euroclone cat# ECS0180L) and 1% antibiotics penicillin-streptomycin, Euroclone cat# ECB3001D) and 1% of L-glutamine (Euroclone cat# ECB3000D). Cells were incubated at 37°C with 5% CO₂. Experiments were performed and repeated at least three times when the cell population reached 60–70% confluence. The “high glucose” concentration in culture was 33 mmol/L d-glucose (EMD Millipore cat# G8644), compared with 5.5 mmol/L d-glucose used as the normal control. The cells were cultivated in high glucose for 2 and 7 days.

Western blot

Cells were dissolved in lysis buffer containing protease inhibitors (Tris HCl pH8 10 mM, NaCl 150 mM, NaF 10 mM, NP40 1%, PMSF 1 mM). Then, the proteins were subjected to 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.22 µm polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk in TBS-T (Tris-buffered pH8/0.15% Tween 20) at room temperature for 1 hour. Then, incubated with primary antibodies diluted in 5% non-fat milk in TBS-T (according to manufacturer instructions) overnight at 4°C. Primary antibodies were: phosphor- NF-κB p65(S276) (Elabscience cat#E-AB-68092), TGF-β (Abcam cat#ab179695), Osteopontin (Abcam cat# ab8448), Galectin 3 (Abcam cat#ab76466), Oxidative stress defense western blot cocktail (Catalase, SOD1, TRX, Abcam cat# ab179843), SOD2 (Abcam cat#ab13533), BAX (Elabscience cat#E-AB-22128), PTEN (Elabscience cat#E-AB-19312), PPAR-γ (cell signaling cat#2443), PPAR-α (Abcam cat#ab227074), GLUT1 (Elabscience cat#E-AB-31556), GLUT4 (Elabscience cat#E-AB-30268), and Vinculin as loading control (Elabscience cat#E-AB-60433). After three washes in TBS-T, the membranes were incubated with corresponding secondary antibodies, horseradish peroxidase-conjugated anti-rabbit IgG, and horseradish peroxidase-conjugated anti-mouse IgG (GE Healthcare), for 1 hour at room temperature. Immunocomplexes were visualized using Clarity Max Western ECL Substrate (BioRad cat#1705062). The molecular weight of proteins was estimated with prestained protein markers (ABM Opti-Protein-Marker cat#G623). Semi-quantitative densitometric analysis was performed using Image J software.

Real-time PCR: miRNA detection

Total RNA, including small RNAs, was isolated and purified from cells using miReasy Mini Kit (Qiagen, Hilden, Germany, cat #217004). First, the RNA concentration and purity were detected using a QIAexpert spectrophotometer (Qiagen, cat #1038703;). Then complementary DNA (cDNA) was synthesized from 10 ng of total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem Lithuania, cat # 4366597) with a specific RT primer (Applied Biosystem, cat # 4440887 has-miR21-5p; cat # 4427975 U6 snRNA) according to the manufacturer's protocol. miRNA expression was measured with a Rotor-Gene Q (Qiagen, cat #R0515102) using PrimeTime Gene Expression Master Mix (IDT, cat #1055772) and the TM primer (Applied Biosystem, cat #4440887 hsa-miR21-5p; cat #4427975 U6 snRNA). The two-step PCR condition was 3 m at 95°C, 45 cycles with 5 s at 95°C, and 30 s at 60°C (10 µl reaction volume and 2 µl cDNA template). All samples were run in triplicate. A threshold cycle (C_t) value was obtained for each

amplification cycle, and ΔC_t was calculated as the C_t difference between target miRNA and U6. miRNA expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Seahorse Analysis

Metabolic status was investigated on a Seahorse XF24 Analyzer (Agilent Technologies) with standard 24-well Seahorse microplates. A Mito Stress Test kit (Agilent Technologies; #103015) was used to assess oxygen consumption ratio (OCR) and ECAR (extracellular acidification rate) after high glucose exposition. In brief, 2×10^4 cells were grown in normal and high glucose medium (for 2 and 7 days) and seeded into Seahorse plates 12 h before analysis. The medium was then replaced by 175 μ l of Seahorse XF DMEM Medium, pH 7.4, containing 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate, and the plate was incubated in a CO₂-free incubator at 37°C for 1 hour to reach temperature and pH equilibrium. The plate was then loaded in XF24 Analyzer, and the injection sequence was programmed as follows: 1st, oligomycin (1 μ M at final concentration); 2nd, carbonylcyanide m-chlorophenylhydrazone (FCCP; 1 μ M at final concentration); 3rd, rotenone, and antimycin A (1 μ M and 0.5 μ M at final concentrations, respectively). Data acquisition was made 100 minutes in 12 steps (represented as dots on OCR curves). Data were analyzed with Wave software (version 2.2.0, Seahorse Bioscience, Agilent Technologies, Santa Clara, CA 95051), following the manufacturer's guidelines. Non-Mitochondrial Oxygen Consumption corresponds to step 12 minimum rate measurement after Rotenone/antimycin A injection; Basal Respiration corresponds to (last rate measurement before first injection) – (Non-Mitochondrial Respiration Rate) and has been calculated as step 3 minus Non-Mitochondrial Oxygen Consumption; Maximal Respiration corresponds to (Maximum rate measurement after FCCP injection) – (Non-Mitochondrial Respiration) and has been calculated as step 7 minus Non-Mitochondrial Oxygen Consumption; ATP production corresponds to (last rate measurement before Oligomycin injection) – (Minimum rate measurement after Oligomycin injection) and has been calculated as step 3 minus step 6; Proton Leak corresponds to (Minimum rate measurement after Oligomycin injection) – (Non-Mitochondrial Respiration) and has been calculated as step 6 minus Non-Mitochondrial Oxygen Consumption; Spare Respiratory Capacity % has been calculated as (Maximal Respiration/ Basal Respiration)*100; Coupling Efficiency has been calculated as (ATP production/Basal Respiration)*100. Data are normalized on control (NG). Experiments were performed in triplicate.

miRNA-21 inhibition LNA

The cells (4×10^5) were cultivated in DMEM/F12 without antibiotics and placed in an incubator for 24h at 37°C. The following day, miRCURY LNA Inhibitor Power (miRCURY LNATM miRNA Power Inhibitor HSA-MIRNA-21-5P 5'-CAACATCAGTCTGATAAGCT-3'; Qiagen, cat #339131 YI04100689-DDA) and its negative control (miRCURY LNATM miRNA Power Inhibitor Control 5'-TAACACGTCTATACGCCCA-3'; Qiagen, cat #339136 YI00199006-DDA) were added directly to the culture medium at a final concentration of 0.2 μ M. 72h after transfection, the cells were subjected to total protein extraction. The transfection efficiency was analyzed by western blot for PTEN, direct protein target of miRNA-21. For Mitochondrial Stress and ATP Production evaluations, AC16 cells were tested 24h, 72h and, 96h following HSA-MIRNA-21-5P inhibition

(0.2 μ M). Data are normalized on negative control (SCR miRNA inhibitor, 0.2 μ M) and compared to vehicle control. Experiments were performed in triplicate.

Results

Effects of high glucose treatment on miRNA-21 expression

The impact of short- (2 days) and long-term (7 days) high glucose treatment on miRNA-21 expression levels was investigated in human cardiomyocytes AC16, exposed to high glucose (HG) (33 mM) for 2 and 7 days.

Cells exposed to high glucose for 2 days (HG 2 days) showed a higher level of miRNA-21 than cells exposed to normal glucose (NG) concentration ($p < 0.05$), while 7 days of high glucose (HG 7 days) treatment induced a reduction of miRNA-21 compared to NG ($p < 0.05$) and HG 2 days ($p < 0.05$) (Fig. 1), suggesting glucose and time-dependent responsiveness of miRNA-21.

Effects of high glucose treatment on pathways involved in cellular damage

- *Oxidative stress and mitochondrial dysfunction*

HG 2 and 7 days of treatment induced an up-regulation of SOD2, the mitochondrial cleaner from ROS ($p < 0.05$) (14) (Fig. 2A). However, after HG 2 days of treatment, no changes were observed in catalase, thioredoxin (TRX), and SOD1 expression, proteins involved in the protection against oxidative stress (15) ($p > 0.05$ vs. NG) (Fig. 2B, C, D). In contrast, after 7 days of HG exposition, all proteins involved in oxidative stress defense were significantly upregulated ($p < 0.05$) (Fig. Fig. 2B, C, D).

- *Inflammation, apoptosis, and fibrosis*

The inflammatory state was evaluated by measuring two principal markers of inflammation, phospho-NF- κ B and TGF- β . No difference in phospho-NF- κ B and TGF- β protein levels between HG 2 days and NG treatment was observed ($p > 0.05$) (Fig. 3A, B). However, HG 7 days treatment-induced activation of inflammatory markers as demonstrated by the upregulation of phospho-NF- κ B and TGF- β protein ($p < 0.05$ vs. NG and HG 2 days) (Fig. 3A, B).

Further, to determine whether HG exposure induced programmed cell death activation, BAX and PTEN were investigated. Specifically, 2 and 7 days of HG treatment caused a decrease and an increase of BAX and PTEN, respectively ($p < 0.05$) (Fig. 3C, D).

Key proteins involved in early and late cardiac fibrosis were also investigated. An increase of early fibrosis marker osteopontin (OPN) was not observed after HG 2 days treatment ($p > 0.05$ vs. NG) but detected after HG 7 days ($p < 0.05$ vs. NG and HG 2 days) (Fig. 3E). No changes in galectin-3 (Gal-3) expression were observed after 2 and 7 days of HG treatment ($p > 0.05$) (Fig. 3F).

Effects of high glucose treatment on cardiac energetic metabolism

- *Glucose transports and peroxisome proliferator-activated receptors*

Intracellular cardiomyocytes metabolism was investigated by measuring the expression of significant glucose transporters, GLUT4 and GLUT1, and regulators of lipid as PPAR- γ and PPAR- α .

GLUT4 expression was down-expressed after 2 days, while an opposite trend after 7 days of glucose treatment ($p < 0.05$) (Fig. 4A). HG 2 days did not affect GLUT1 level ($p > 0.05$) while HG 7 days treatment induced a reduction in GLUT1 expression compared to NG ($p < 0.05$) and HG 2 days ($p < 0.05$) (Fig. 4B).

Two days of HG treatment was associated with a shift toward myocardial glucose utilization as demonstrated by the upregulation of PPAR- γ and the reduction of PPAR- α protein levels ($p < 0.05$ vs. NG) (Fig. 4C, D). However, after HG 7 days of treatment, PPAR- γ and PPAR- α protein levels returned to the baseline condition ($p < 0.05$ vs. HG 2 days) (Fig. 4C, D).

- *Effect on mitochondrial respiration and ATP production*

Exposure of cardiomyocytes to high glucose modulated mitochondrial respiration in a time-dependent manner, showing attenuated response to β -oxidative stimulation at 7 days compared to 2 days (Fig. 5 A). Oxygen Consumption Rate (OCR) increased basal respiration at HG 2 days while the 7 days long-term had an opposite result (Fig. 5B).

Zooming in metabolic parameters, cardiomyocytes showed a more efficient maximal respiration under stimulation of 2 days compared to the drastic reduction observed after HG 7 days (Fig. 6 A). Furthermore, the severe drop of ATP production and spare respiratory capacity confirmed that solely the long-term exposition to HG 7 days induced the depletion of the cellular energy function, which is unaffected until HG 2 days (Fig. 6B, C). On the contrary, HG 2 days caused proton leak increase accompanied by the energy potential dissipation (Fig. 6D). Non-mitochondrial oxygen consumption was reduced at 7 days (Fig. 6E), according to the spare respiratory capacity reduction, representing a particularly robust functional parameter to evaluate mitochondrial reserve. Moreover, HG determined a reduction of coupling efficiency only when the induction time is 2 days (Fig. 6F).

LNA-anti-miRNA-21

To examine the implication of miRNA-21 in the short and long-term high glucose treatment, LNA-modified anti-scrambled and anti-miRNA-21 oligonucleotides were transfected into the AC16 cell line for 72 h and proteins involved in cellular damage and energetic metabolism were investigated.

The cellular damage evaluation revealed that the treatment with LNA-anti-miRNA-21 increased catalase protein levels ($p < 0.05$ vs. NG and anti-scrambled) (Fig. 7B). However, no difference in other proteins involved in oxidative stress response was observed.

Moreover, it also caused activation of inflammation and apoptosis markers, as demonstrated by the upregulation of phospho-NF- κ B, TGF- β , PTEN, and PDCD4 ($p < 0.05$ vs. NG and anti-scrambled) (Fig. 7E-7F).

H).

To confirm the protective role of miRNA-21 per se in the cardiac model, discrete changes in real-time cellular bioenergetics were measured following LNA-mediated miRNA-21 inhibition (supplementary files Fig 1 Panels A-H). In particular, the effect of LNA-anti-miR-21 on AC16 metabolism was minimal after a time of 24h from the induction but became evident at a longer time, thus confirming a pivotal impact of miRNA-21 as determinant of cardiac energy metabolism.

Discussion

In human AC16 cardiomyocytes, we provided evidence that: i) short term (48h) high glucose treatment induced a significant increase in miRNA-21 expression and function, which was associated with increased hydrogen ion flux and energy potential dissipation without any change in energy production or increase in the expression of cellular damage genes; ii) the inhibition of miRNA-21 function induced the activation of inflammation, apoptosis pathways and compromises mitochondrial function as demonstrated by the incapacity to answer energy demand; iii) long term (7days) myocardial cells exposition to high glucose treatment evoked a decrease in miRNA-21 levels combined to an increased expression of cellular damage-related genes. In addition, a parallel depletion of the cellular energy function with secondary impairment of physiological mitochondrial function was also observed.

These results demonstrate that in human cardiomyocytes, the cardioprotective effect of miRNA-21 towards high-glucose treatment related damage is dose and time-dependent and indicate, for the first time, a potential role of miRNA-21 in molecular mechanisms underlying the myocardial metabolic flexibility, i.e., the heart's ability to adjust its substrate preference to a short-term external metabolic insult to sustain an adequate ATP production for correct cardiac contractile function.

The miRNA-21 plays a key role in many biological processes, and it is expressed in cardiomyocytes, fibroblasts, and endothelial cells, where it regulates apoptosis, inflammation, fibrosis, and metabolism. However, its role in cardiovascular diseases is still controversial (13, 16). Several previous data showed miRNA-21 to display in cardiomyocytes a protective effect toward oxidative stress via activation of PDCD4 and AP-1, two members involved in the apoptosis cascade pathway (5), and attenuates the injuries induced by palmitate (17). Indeed, miRNA-21 knockout mice displayed fibrosis and cardiac hypertrophy in response to various cardiac stresses (18). In contrast, other studies demonstrated that a high glucose treatment increased the miRNA-21 expression in cardiac fibroblast, inducing fibrosis, hypertrophy, and cardiac dysfunction (17, 19) and that elevated myocardial and bloodstream miRNA-21 levels significantly correlates with the severity of left ventricular fibrosis in aortic stenosis patients.

Interestingly, miRNA-21 has been recently demonstrated to play a critical role in the "diseased heart" by regulating myocardial energy metabolism (20). In normal conditions, to respond to the continuous demand of ATP, the heart can metabolize different substrates through mitochondrial oxidative phosphorylation, like as glucose, fatty acids, amino acids, and lactate. The main regulatory mechanisms include the uptake of substrates into cardiomyocytes through facilitated diffusion mediated by

membrane-associated proteins (CD36 for fatty acids, GLUT1, and GLUT4 for glucose) and mitochondrial oxidation (21, 22). Even though the heart uses fatty acids as the primary substrate in normal conditions, a specific and delicate regulated balance exists between substrates. Increasing evidence suggests that the heart works better when it uses substrates mixture, especially fatty acids and glucose (23, 24). However, when the balance of substrates is a slant towards the predominant utilization of fatty acids or glucose, it is linked to impaired cardiac contractile function. In addition, the preferred use of one substrate regarding another increases the risk that the heart suffers from fuel toxicity, i.e., lipo- or glucotoxicity, conditions that elicit significant impairments of cardiac functioning (22, 25, 26). Specifically, glucose 6-phosphate due to a mismatch with glucose oxidation during excess glucose availability, leading to mTOR activation and increased protein synthesis resulting in cardiac hypertrophy (22, 27). In addition, long-term glucose supply and utilization are linked to a fetal transcriptional program in adult cardiomyocytes. This fetal switch is maladaptive, whereby contractile force decreases and induces ROS formation and increased glycation (28).

"Metabolic flexibility" is the capacity of the heart to adapt its substrate preference to short-term detrimental stimuli to maintain an adequate ATP production for optimal cardiac contractile function. However, persistent metabolic insult occurs when the balance of metabolic substrates is shifted towards the utilization of one substrate (either glucose or fatty acids), impairing metabolic flexibility and leading to suboptimal ATP production and impairing contractile function. Indeed, as ATP production is reduced, heart failure progresses overall (22).

However, the mechanisms underlying such metabolic flexibility and what happens during long-term increases in glucose supply and utilization are not yet fully understood.

Interestingly, in our study, short-term (2 days) exposure of cardiomyocytes to high glucose treatment elicited an up-regulation of miRNA-21, which in turn results in a protective action toward oxidative stress, inflammation, fibrosis, apoptosis. Indeed, cardiomyocytes responded to short-term high glucose concentration with a decrease of GLUT4 and PPAR- α , and increased PPAR- γ expression for downgrading glucose uptake and rising its metabolism to prevent high glucose insult. Furthermore, after a short-time human cardiomyocyte exposition to high glucose, more O₂ consumption and H⁺ dissipation was not translated in ATP production. More intriguing, when cardiac injury persisted for a long time (7 days), the miRNA-21 levels decreased, cardiomyocytes could not protect themselves, and functional failure occurred as demonstrated by oxidative stress, inflammation, apoptosis, and fibrotic markers. Moreover, when miRNA-21 is blocked with its LNA-antagomi-RNA, basal respiration, maximal respiration, and ATP production processes are impaired in AC16 cells in a time-dependent manner.

These results suggest that cardiomyocytes might activate a compensative way to respond to the presence of abundant glucose substrate, probably through up-regulation of miRNA-21. In parallel, miRNA-21 blockage alone impedes the correct metabolic flux inside cardiomyocytes. In agreement, a recent study provides direct evidence that modulation of miR-21 levels can impact substrate utilization and mitochondrial respiration in H9C2 cells. The different substrate availability can further modulate this

effect (29). Furthermore, in our study, miRNA-21 inhibition activated inflammation and apoptosis pathways, compromised mitochondrial function (as demonstrated by the reduction in O₂ consumption and ATP production), thus inducing a functional failure with the inability to meet energy demand. These findings strengthen the idea that miRNA-21 presence is necessary for physiological conditions especially when the metabolic burden shifted for availability/accumulation of a predominant substrate (here glucose).

Noteworthy, long-term exposure to high glucose treatment, a condition associated with downregulation of miRNA-21, definitively compromises the cellular metabolism as demonstrated by the reduction of spare capacity, indicating that cardiomyocytes lost flexibility and cell fitness (30). These data are also supported by the decrease in O₂ consumption, ATP production, and the increase of H⁺ dissipation with consequent activation of antioxidant defense. Moreover, the up-regulation of PPAR-α and the down-regulation of PPAR-γ suggest that cardiomyocytes try to revert the metabolic imbalance in favor of fatty acid oxidation. Still, this effort helps to increase ROS generation to toxic levels, leading to subsequent activation of the antioxidant defense system (31).

These results highlight the complexity of factors that regulate metabolic pathways in the cardiac cells and suggest that, under hyperglycemic conditions, the cardiomyocytes, before irreversible damage, realize an early and late adaptation that recalls the two preliminary phases of diabetic cardiomyopathy. Indeed, in an early phase, the cell recognizes a protective mechanism against the high exposure of high glucose-mediated by miRNA-21; subsequently, as a consequence of miRNA-21 downregulation, the cell cannot counteract the damage and goes into functional metabolic deficiency. Moreover, even when glucose is not the primary energy source, the inhibition of miRNA-21 function alone shows time-dependent effects. Indeed, at 24h from the LNA treatment, cardiomyocytes maintain the metabolic flow while a decrease in the total cellular ATP still uncompensated by the non-mitochondrial oxygen consumption pathways over a longer time incubation was observed. As a whole, our data underline the importance of assessing alteration in mitochondrial respiration in addition to genes expression and points out the crucial role of miR-21 as a new potential target for metabolic intervention aimed at securing sufficient ATP production to sustain organ function both in response to short- and long-term metabolic insult.

Conclusions

In human cardiomyocytes cell line our data demonstrated that the reliance on glycolytic and fatty acid oxidation pathways is modulated by the abundance of miRNA-21 within the cells, regulating mitochondrial respiration and the ability of cells to select the most appropriate substrate for ATP production in a given environment. Therefore, we contribute to solving the paradigm on the role of miRNA-21, showing that it takes part in the first defense mechanism against cardiac insult and its cardioprotective effect depends on the time of exposure to the injury.

Abbreviations

AP-1: Activator Protein-1

ATP: Adenosine Triphosphate

BAX: BCL-2-like protein 4

DC: Diabetic Cardiomyopathy

ERK: Extracellular signal-regulated kinase

Gal-3: Galectin-3

MAPK: mitogen-activated kinase pathways

miRNA: micro-RNA

NF- κ B: Nuclear factor kappa B

NO: Nitric Oxide

OCR: Oxygen Consumption Rate

OPN: Osteopontin

PDCD4: downregulating Programmed Cell Death 4

PI3K: phosphoinositide 3-kinase

PPAR: Proliferator-activated receptor

PTEN: Phosphatase and Tensin Homolog,

ROS: Reactive Oxygen Species

TGF- β : Transforming growth factor beta

TLR4: Toll-like receptor 4

TNF- α : Tumor Necrosis Factor-alpha

TRX: Thioredoxin

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All other authors declare that they have no competing interests.

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Authors' contributions

G.P., L.A., M.B. and R.F. designed the study and critically revised the manuscript, and G.P. is the guarantor of this work and is responsible for its integrity. L.S. and R.B. wrote the manuscript. L.S., R.S., U.C., R.A.F., N.D.G., S. performed the experiments and analyzed the data. All authors read and approved the final manuscript.

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Figures

Figure 1

miR-21 expression levels. Data are mean $\pm$ SEM. * $P < vs$ NG; ** $P < vs$ HG 2 days

Figure 2

Cellular response to short and long-term high glucose treatment: Western blot analysis of proteins involved in oxidative stress. AC16 cell line were exposed for 2 and 7 days at high glucose (33mM). Band quantification was quantified with ImageJ software. Data are mean $\pm$ SEM. * $P < vs$ NG; ** $P < vs$ HG 2 days

Figure 3

Cellular response to short and long-term high glucose treatment: Western blot analysis of proteins involved in inflammation, apoptosis, and fibrosis. AC16 cell line were exposed for 2 and 7 days at high glucose (33mM). Band quantification was quantified with ImageJ software. Data are mean $\pm$ SEM. * $P < vs NG$; ** $P < vs HG 2 days$

Figure 4

Cellular response to short and long-term high glucose treatment: Western blot analysis of proteins involved in metabolism. AC16 cell line were exposed for 2 and 7 days at high glucose (33mM). Band quantification was quantified with ImageJ software. Data are mean $\pm$ SEM. * $P < vs$ NG; ** $P < vs$ HG 2 days.

Figure 5

Main mitochondrial respiratory parameters following HG exposure: Image shows: **A)** time course of Oxygen Consumption Rate (OCR) of AC16 cardiomyocytes in normal glucose (NG), in high glucose at 2 days (HG 2 days) and in in high glucose at 7 days (HG 7 days). **B)** Basal respiration. Values are reported as OCR (pmol/min) normalized on NG. Statistical significance has been calculated as t-test and reported * $P < vs$ NG; ** $P < vs$ HG 2 days.

Figure 6

Bioenergetics parameters associated with HG exposure: Histograms show the normalized interpolation data from OCR of AC16 cardiomyocytes in NG, HG 2 days and HG 7 days. A) Maximal Respiration, B) ATP production, C) Spare Respiratory Capacity %, D) Proton Leak, E) Non-mitochondrial Oxygen Consumption and F) Coupling Efficiency %. Statistical significance has been calculated as t-test and reported * $P < \text{vs NG}$; ** $P < \text{vs HG 2 days}$.

Figure 7

Cellular response to LNA-anti-miR-21: Western blot analysis of proteins involved in cellular damage and energetic metabolism. Band quantification was quantified with ImageJ software. Data are mean $\pm$ SEM. * $P < 0.05$ vs CTRL.

Supplementary Files

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