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Review Article

WNT3A Aggravates Oxygen-Glucose Deprivation-Induced Injury in H9C2 Cardiomyocytes via Inhibition of SIRT3

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Abstract

Ischemic injury is a major cause of several cardiovascular diseases such as myocardial infarction, cardiac hypertrophy, and ventricular remodelling. WNT signaling has been shown to be a key regulator of cell growth, injury, and repair. Using the *in vitro* Oxygen and Glucose Deprivation (OGD) model to mimic ischemic condition, we found that WNT3A over expression could enhance the OGD-induced injury in H9C2 cardiomyocytes. WNT3A significantly decreased cell viability, promoting the generation of apoptotic cells and increasing autophagosome formation. Coincidently, WNT3A over expression up regulated the expression of Caspase-3 in mRNA level and the amount of cleaved PARP-1 in protein level. In addition, we observed that WNT3A aggravated the OGD-induced mitochondrial dysfunction and cytosolic release of cytochrome C. Furthermore, we identified SIRT3, a mitochondrial NAD+ - dependent deacetylase modulating mitochondrial metabolism and homeostasis, as a main target affected by WNT3A over expression. WNT3A inhibited the expression of SIRT3. The effects of WNT3A over expression were effectively reversed by the addition of SFRP4, a natural antagonist to the WNT ligands. Conversely, SIRT3 over expression could repress WNT3A expression and ameliorate OGD-induced mitochondrial dysfunction. Furthermore, the dysregulation of WNT3A and SIRT3 was measured in the acute myocardial infarction model. Overall, our findings suggest that the WNT3A -SIRT3 regulatory axis might be a potential target for cell protection in cardiac ischemia and hypoxia.

Keywords: Ischemic heart disease; WNT3A ; Oxygen and glucose deprivation; Apoptosis; SIRT3; SFRP4

Introduction

Ischemic Heart Disease (IHD), such as Myocardial Infarction (MI), poses a major threat to human health and causes extensive damages to myocardial tissues, remaining a major cause of disability and death worldwide. This heart problem occurs when the heart muscle receives insufficient blood flow. It closely links with the pathological process of atherosclerosis. The long-term accumulation of cholesterol-rich plaques in the coronary arteries gives the rise of narrowed heart arteries. When coronary arteries are narrowed to certain extent, inadequate blood and oxygen reaches the myocardium. The disproportion between myocardial oxygen supply and demand results in myocardial hypoxia and induces the death of cardiomyocytes. Both necrosis and apoptosis play an important role in the process of tissue damage subsequent to myocardial ischemia [1].

Oxygen-Glucose Deprivation (OGD) is widely used to create the cellular model of ischemic heart injury *in vitro* [2,3]. In order to perform OGD, primary myocardial cells or H9C2 cardiomyocytes are incubated in a glucose-free medium under a deoxygenated atmosphere. OGD is a simple yet highly useful model, not only for the illumination of the role of crucial cellular and molecular mechanisms underlying cardiac ischemia, but also for the development of possible prevention strategies.

Many mechanisms that include oxidative stress, calcium overloading, and mitochondrial dysfunction, are involved in OGD-induced myocardial injury and eventually contribute to cell apoptosis or necrosis. Otherwise, many inflammatory cytokines are released and participate in ischemic heart injury, such as tumor necrosis factor-α (TNF-α), Interleukin (IL)-1β, IL-6, and IL-8 [4]. Abnormal WNT signaling is also associated with the process of ischemic heart disease and myocardial infarction [5,6]. The WNT family, involving 19 distinct WNT ligands and 10 Frizzled (Fzd) receptors, plays key roles in the embryogenesis and functioning of various organ systems. WNT proteins are a conserved family of secreted, extensively palmitoylated glycoproteins. WNT ligands function through binding with a heterodimeric receptor complex comprised of seven-transmembrane Fzd receptors and coreceptors low-density LRP5/6. Secreted Frizzled-Related Protein (Sfrp) is a secreted family of endogenous WNT antagonists, containing five members (SFRP1–5) that block WNT/Fzd interactions. WNT signaling involves two principal cascades, β-catenin dependent (canonical), and β-catenin independent (non-canonical) pathways. β-catenin, the critical downstream regulator of the canonical WNT pathway, is activated when WNT1, WNT 2a, WNT3A, or WNT8 ligands bind to Fzd receptors. β-catenin together with the transcription factors TCF/LEF1 favors the transcription of target genes such as *Cyclin D1* and *c-Myc*. Thereby, canonical WNT signaling is extensively involved in diverse cell events such as proliferation, motility, differentiation, inflammation, and apoptosis.

WNT signaling is mostly quiescent in the healthy adult organs

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but the reactivation of WNT signaling is frequently observed under pathological conditions. It is well known that dysregulation of WNT signaling is related with cardiac hypertrophy and fibrosis, myocardial infarction, heart failure and arrhythmias [5,6]. However, it is highly controversial that WNT signaling may be either beneficial or harmful effects for ischemic heart disease. As different WNT ligands and receptors may exert distinct effects. In this study, we investigated the role of WNT3A in the OGD model of H9C2 cardiomyocytes. Our results indicated that WNT3A overexpressing sensitized H9C2 cardiomyocytes to OGD-induced apoptosis. By comparsion, SFRP4 anatognized WNT3A and increased cell viability in H9C2 cells subjected to OGD. Mechanistically, WNT3A inhibited the expression of SIRT3, a crucial protein in mitochondrial integrity and function. Importantly, SIRT3 over expression exerted negative effects on WNT3A expression, and facilitated cell survival under the OGD condition. The alteration of WNT3A and SIRT3 expression in the mouse myocardial infarction model was further validated. These data suggest that the mutual interaction of WNT3A and SIRT3 is associated with the pathologic process of cardiac ischemia.

Materials and Methods

Cell lines and culture

The H9C2 cells, a cardiomyocyte cell line originally derived from the rat left ventricle, were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were cultured in high glucose (4500 mg/L) Dulbecco's Modified Eagle Medium (DMEM) supplemented with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% fetal bovine serum (FBS, GIBCO). The cells were maintained at 37 °C in a humidified incubator with 5% CO_2. The medium was displaced every 2 to 3 days, and cells were subcultured or subjected to experimental procedures when developed to 80% to 90 % density.

Plasmid constructs and transfection

Expression vectors for WNT3A and β-gal were a kind gift from Dr. Roel Nusse (Stanford University School of Medicine) and Dr. Zhiqiang Liu (College of Basic Medicine, Tianjin Medical University) respectively [7]. Plasmid DNA for transfection was prepared using a Hi Speed Plasmid Midi kit (Qiagen, Australia). Transient transfection was performed using Lipofectamine2000 (Invitrogen, USA) with encoding plasmids. 48 h after transfection, 1 mg/mL G418 was added into the culture medium to eliminate untransfected cells. After antibiotic selection for 10 days, transfectants were pooled and expanded in 500 μg/mL G418. H9C2 cells with stable expressing WNT3A were used for further experiments and the pcDNA3.1/β-gal transfected parental cells were used as negative control.

Oxygen glucose deprivation (OGD)

As described previously, the cell culture medium was replaced with serum-free and glucose-free DMEM, and then H9C2 cells were exposed to hypoxic conditions (5% CO_2, 94% N_2, and 0.5% O_2) in a 37°C incubator at for the indicated time as the OGD group [3]. The environment mimicked the *in vivo* conditions of myocardial infarction. Cells were treated for various periods in the presence or absence of recombinant SFRP4 (R&D Systems, USA).

Cell viability assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8; Dojindo, Japan) according to the manufacturer's instructions. Briefly, H9C2 cells were seeded in 96-well plates at 4×10^3 cells/well. After 24 h, cells were exposed to OGD treatment. At the end of the treatment period, 10 μL CCK-8 solutions were administered into each well and the cells were incubated for 2 h at 37°C. The absorbance of each well at 450 nm related to the reference absorbance at 630 nm was measured using a micro plate reader (Bio-Rad, USA). The cell viability percentage was calculated using the following formula: % cell viability=(mean absorbance in the test wells)/(mean absorbance in the control well) × 100. Each experiment was repeated five times and the data were expressed as a percentage of the control.

Apoptosis assay

Using Annexin V-FITC and Propidium Iodide (PI) staining (Keygen Biotech, China), cell apoptosis was assessed by flow cytometry according to the manufacturer's instructions. After incubation for the indicated treatment, 1×10^5 cells in 500 mL of binding buffer were mixed with 5 mL of Annexin V-FITC, and then 5 mL of PI (50 mg/mL) was added to the cells, which were incubated at room temperature in the dark for 1 h. The stained cells were analyzed using a FACS Calibur instrument (BD Biosciences, USA). The data of flow cytometry was analyzed with Cell Quest 3.0 software (BD Biosciences, USA). Cells that were stained negatively with Annexin V and PI were considered viable cells. Early apoptotic cells were positive for Annexin V and negative for PI, and late apoptotic cells were positive for Annexin V and PI.

Detection of mitochondrial transmembrane potential

The mitochondrial membrane was monitored using rhodamine 123 fluorescent dye (Ex/Em=485 nm/535 nm; Sigma), a cell-permeable cationic dye, which preferentially enters into mitochondria due to the highly negative Mitochondrial Membrane Potential (MMP). Depolarization of MMP results in the loss of rhodamine 123 from the mitochondria and a decrease in intracellular fluorescence. In brief, H9C2 cells under different treatments were incubated with rhodamine-123 (0.1 μg/ml) at 37°C for 30 min. The intensity of rhodamine-123 staining was determined using a BD flow cytometer.

Cell extraction and western blotting

H9C2 cells were collected, washed twice with PBS, and then lysed for 30 min on ice in a lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM EDTA, 1% Triton X-100, 0.5% SDS and protease inhibitor cocktail). For subcellular fractionation, H9C2 cells were harvested in isotonic mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM Hepes, pH 7.5) and then homogenized for 30 to 40 strokes with a Dounce homogenizer. Cell lysate was centrifuged at 500 g for 5 min at 4°C to eliminate nuclei and unbroken cells. Supernatant was then centrifuged at 10,000 g for 30 min at 4°C to obtain mitochondrial fraction, and the resulting supernatant was stored as the cytosolic fraction. The protein concentration in cell lysate was measured by using Protein Quantification kit from Bio-Rad. Total 30 μg proteins were loaded onto an SDS-PAGE gel. After transferring and blocking, the blots were probed with various antibodies (anti-PARP-1, anti-SIRT3, anti-β-actin, and anti-cytochrome c, Santa Cruz Biotechnology). The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using an enhanced chemiluminescence system (Millipore, USA).

Real-Time PCR

Total RNA was extracted from the H9C2 cells with different treatments using TRIzol reagent (Invitrogen, USA) and then reverse transcribed and synthesized into cDNA using Super Script First Strand Synthesis system (Invitrogen). Real-Time PCR was performed with Power SYBR Green PCR Master mix (Applied Biosystems, USA). The threshold cycle (Ct) was obtained from

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triplicate samples and averaged. The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and standardized by *Gapdh* gene. Primers used for PCR amplification in H9C32 cells were: *WNT3A*, 5'-ATTTGGAGGAATGGTCTCTCG-3', 5'-GCAGGTCTTCACTTCGCAAC-3'; *SIRT-3*, 5'-CATGGCCCTGAAATACGAAGTC-3', 5'-GCAGGCCTGAATGATGAAGAGTTT-3'; *Gapdh*, 5'-AACGACCCCTTCATTGACCTC-3', 5'-CCTTGACTGTGCCGTTGAACT-3'. Primers used for PCR amplification in mice heart tissue were: *WNT3A*, 5'-ATTTGGAGGAATGGTCTCTCG-3', 5'-GCAGGTCTTCACTTCGCAAC-3'; *SIRT-3*, 5'-CATGGCCCTGAAATACGAAGTC-3', 5'-GCAGGCCTGAATGATGAAGAGTTT-3'; *Gapdh*, 5'-AACGACCCCTTCATTGACCTC-3', 5'-CCTTGACTGTGCCGTTGAACT-3'. Three independent experiments were performed in triplicate.

Immunofluorescence staining

After OGD treatment, H9C2 cells were fixed in 4% formaldehyde for 10 minutes. For fluorescence staining, the samples were treated in 0.5% (V/V) Triton X-100 for 15 min and blocked with 10% BSA for 30 min at 37°C. Then cells were incubated overnight at 4°C with anti-LC3 antibody, followed by incubation with TRITC-conjugated secondary antibody for 30 min at room and nucleus counterstaining with Hoechst 33342. Image was captured using a fluorescence microscope (model IX71; Olympus).

Model of MI

Myocardial ischemia was induced in the mouse by ligating the left coronary artery, a method previously used [8,9]. Male C57BL/6 mice (8 weeks to 10 weeks old) were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China). Mice anesthetized with sodium pentobarbital (80 mg/kg, i.p.) were intubated through a tracheotomy, and ventilated with 3 cm H_2O positive-end expiratory pressure. Adequacy of anaesthesia was monitored using the corneal and withdrawal reflexes. Ventilation frequency was kept at 110 breaths per minute with a tidal volume between 135 μL and 150 μL. After opening the chest, the Left Anterior Descending Coronary Artery (LAD) was surrounded by a 7-0 Prolene suture and the coronary artery was occluded by pulling on the suture tightly. Regional ischemia was confirmed by visual inspection of pale color of the myocardium and the elevation of ST segment on the electrocardiogram. Sham operation involved an identical procedure, except that the suture was passed through the myocardium without tying, and was removed. The chest was closed in two layers, and the animals were allowed to recover. Mice were reanesthetized with methoxyflurane and killed 18 h after coronary ligation. All of the animal experiment procedures were approved by the Animal Care and Use Committee of Henan University.

Measurement of infarct size

Triphenyl Tetrazolium Chloride (TTC) staining was used to determine myocardial infarct size. Infarct size was assessed after the end reperfusion. Hearts were excised and sliced. The slices were incubated in 1% TTC in sodium phosphate buffer at 37°C for 20 min and fixed with 10% formalin at room temperature. Infarct size was measured using a microscope (model IX71; Olympus).

Statistics

Data are presented as mean±Standard Deviation (SD). Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL). Statistical significance was determined using a two-tailed Student's t-test. In all experiments, $P<0.05$ was considered statistically significant.

Results

OGD upregulatesWNT3A expression

WNT3A is an important WNT ligand involved in cell injury and inflammation. Its exact roles during ischemic heart disease and myocardial infarction still need to be unravelled. In this study, we established an *in vitro* model of Oxygen Glucose Deprivation (OGD), showing similarities with the *in vivo* models of heart ischemia. OGD treatment triggered an increase of *WNT3A* expression in mRNA level. 24 h post OGD treatment, the transcript level of *WNT3A* was enhanced by approximately 5.6-fold. Meanwhile, the protein abundance of WNT3A also exhibited similar alteration. This result suggests that WNT3A is involved in myocardial injury caused by OGD.

WNT3A enhances OGD-induced cell injury in H9C2 cardiomyocytes

Subsequently, we established H9C2 cardiomyocytes overexpressing WNT3A or β-gal proteins. As a consequence of WNT3A overexpression, the expression of β-catenin and c-Myc was greatly augmented in H9C2 cardiomyocytes. Subsequently, H9C2 cells were subjected for OGD treatment to mimic insufficient blood flow in ischemic heart disease. The viability of H9C2 cells decreased gradually during the course of OGD, with about 63.5% of H9C2 cells overexpressing β-gal survived at 12 h. Compared with β-gal group, WNT3A over expression significantly decreased the viability of H9C2 cardiomyocytes. The relative cell viability in WNT3A over expressing group declined to 32.4% after OGD treatment for 12 h. Under normoxic and nutrient condition, H9C2 cardiomyocytes displayed a spindle shape with a clear integral structure and edge. In contrast, morphological changes of apoptosis, such as cell shrinkage and rounding, were observed after OGD exposure. Apparently, WNT3A overexpression aggravated the morphological alteration of H9C2 cardiomyocytes and facilitated the occurrence of cell debris under the OGD condition. Taken together, these data indicated that WNT3A overexpressing in H9C2 cells exerts inhibitory effects on cell viability in the OGD model.

WNT3A promotes cell apoptosis and autophagy

Subsequently, the effect of WNT3A on cell survival was further quantified with flow cytometry by double-staining of Annexin V-FITC and PI. WNT3A overexpression for 48 h initiated cell apoptosis moderately. After OGD treatment for 12 h, the amount of positive apoptotic H9C2 cardiomyocytes stained with Annexin V-FITC reached 37.7% and 57.7% in β-gal and WNT3A groups, respectively. Necrotic cells with Annexin V-positive/PI-negative were nearly negligible. Meanwhile, we examined the combined effect of WNT3A overexpression and OGD treatment on the expression of *Caspase-3*, a critical executor of apoptosis. A higher mRNA level of *Caspase-3* gene was observed in OGD-treated WNT3A group compared with β-gal group. Since Caspase-3 cleaves multiple vital cellular proteins such as poly (ADP-ribose) polymerase-1 (PARP-1), an active feature of cell apoptosis. Consistently, the amount of cleaved PARP-1 was significantly higher in WNT3A -overexpressing cells after OGD treatment for 12 h. These data suggest that WNT3A enhances cell apoptosis, thereby deteriorating OGD-induced H9C2 cell injury. On

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the other hand, we also found that WNT3A overexpression favored the formation of cell autophagy. In this study, LC3 staining was utilized to label the autophagosome in autophagic cells. In the OGD-treated control group, LC3 staining was predominantly diffuse in the β-gal overexpressing cells and it was difficult to characterize LC3-positive puncta. However, dense LC3-positive puncta were easily observed in the cytoplasm surrounding the nucleus of WNT3A -overexpressing cells following OGD treatment. Collectively, WNT3A overexpression may cause cell injury via inducing cell apoptosis and autophagy in the OGD condition.

WNT3A decreases SIRT3 expression and aggravates mitochondrial dysfunction

Mitochondrial Membrane Potential (MMP) is fundamental to the maintenance of mitochondrial physiological function and cellular bioenergetic homeostasis. The collapse of mitochondrial integrity is the initial step to trigger the intrinsic pathway-dependent apoptosis. The leak of apoptogenic factors from the mitochondria into the cytoplasm, such as Cytochrome C (Cyt-C), is a common feature of mitochondrial disorder and cell apoptosis. We therefore investigated possible loss of MMP using rhodamine 123 staining followed by flow cytometry. The results showed that OGD administration significantly decreased the MMP of H9C2 cardiomyocytes. Relatively, cellular MMP was lower in WNT3A -overexpressing cells than in β-gal overexpressing cells. In parallel, OGD-induced cytosolic release of Cyt-C was markedly enhanced in WNT3A group. These findings support that WNT3A overexpression prompts cardiomyocyte apoptosis through the mitochondria-dependent apoptotic pathway.

SIRT3 is the main mitochondrial NAD+-dependent deacetylase [10]. SIRT3 regulates the enzymatic activity of diverse proteins involved in mitochondrial physiological function, such as ricarboxylic acid cycle, fatty acid oxidation, telectron transport chain, and oxidative phosphorylation. It has been demonstrated that SIRT3 plays a critical role in maintaining metabolic and redox balance in the mitochondria under physiological and pathological conditions. The complicated relationship of SIRT3 with ischemic heart disease, myocardial infarction, ischemia-reperfusion injury, cardiac repair and remodelling has attracted much attention in the recent years. Herein, we found that WNT3A overexpression directly reduced the expression of SIRT3 in H9C2 cardiomyocytes. SIRT3 expression was further inhibited in the OGD condition. This mechanism may sensitize H9C2 cells to OGD-induced cell injury. Thus, we identify SIRT3 as a target molecule affected by WNT3A .

SFRP4 antagonizes WNT3A effect to relieve cell injury

Secreted Frizzled-Related Proteins (SFRP1-5) are a family of glycoproteins that function as natural antagonists to the WNT ligands. Previous study revealed that recombinant SFRP4 abrogated the stimulatory effect of WNT3A on the proliferation and migration of malignant mesothelioma cells [11]. Next, we evaluated the effects of WNT3A blockade using recombinant SFRP4 protein. Elevated concentrations of SFRP4 protein were supplemented into the culture medium prior to the onset of OGD. Indeed, recombinant SFRP4 at 200 mg/mL effectively inhibited the pro-apoptotic activity of WNT3A and rescued cell death in the OGD condition. Consistently, SFRP4 addition also decreased the amount of Cyt-C in the cytosolic fractions of WNT3A overexpressing cells, thereby contributing to the maintenance of mitochondrial homeostasis. Further investigation showed that recombinant SFRP4 could effectively rescued the expression of SIRT3 in WNT3A group. Therefore, our results suggest that the antagonism of WNT3A by SFRP4 can render H9C2 cardiomyocytes with more resistance to OGD condition. It may partially attribute to the up regulation of SIRT3 in the presence of SFRP4.

SIRT3 overexpression reverses WNT3A up regulation and aggravates mitochondrial dysfunction

As WNT3A can reduce SIRT3 expression and aggravate mitochondrial dysfunction. Next, we examined the effects of SIRT3 overexpression on WNT3A expression and cell damage. SIRT3 overexpression effectively decreased the amount of cleaved PARP-1 in H9C2 cardiomyocytes after OGD treatment for 12 h. In parallel, SIRT3 overexpression partly reversed OGD-induced cardiomyocyte apoptosis and the release of Cyt-C from mitochondria to the cytoplasm. Importantly, SIRT3 overexpression was capable of interrupting WNT3A up regulation under the OGD condition. Therefore, our results suggest that SIRT3 can antagonize the effect of WNT3A and exert a protective action on cardiomyocyte survival in the OGD condition.

WNT3A and SIRT3 expressions were changed in a mouse MI model

Subsequently, we examined the expression of WNT3A and SIRT3 in acute myocardial infarction. The successful induction of MI was verified by continuous electrocardiograph monitoring. S-T segment elevation after ischemia was observed. Then, TTC staining showed a significant increase in infarct size after acute MI. To ascertain the dysregulation of WNT3A and SIRT3, western blotting was performed to evaluate these samples from the sham and acute MI groups. Our data showed that acute MI greatly decreased SIRT3 expression, meanwhile mildly enhanced the expression of WNT3A both at the mRNA level and protein level. It implicates that the dysregulation of WNT3A and SIRT3 is associated with acute MI-induced injury.

Discussion

Up to now, emerging evidences suggest an important role for the WNT family in regulating cardiac injury, repair and remodelling events [5,6]. In response to pathological stress, the heart reactivates several signaling cascades that traditionally were thought to be operational only in the development of heart, such as WNT pathway. The canonical WNT/β-catenin pathway has a conserved role in vertebrate heart development, restricting the proliferation and commitment of embryonic stem cells toward functional cardiomyocytes. Indeed, WNT/β-catenin signal exerts a stage-specific, at least biphasic role during different aspects of cardiac development [12]. The WNT family comprises 19 different ligands and has been implicated in various cellular processes including apoptosis and cell survival. However, the precise cellular effect depends on the dose and type of WNT ligand, target cells as well as environmental context. In this study, we investigated the effect of WNT3A in the OGD model of H9C2 cardiomyocytes imitating ischemic condition. Furthermore, our results revealed that WNT3A could repress the expression of SIRT3 to aggravate cell injury, thereby establishing a link between WNT3A and SIRT3 in the pathologic process of cardiac ischemia.

Ischemic heart disease, such as myocardial infarction, is one of the most frequent acute cardiovascular events. In any case, owing to the interrupted blood flow the ischemic region of the heart is deprived of oxygen and nutrients, leading to cell injury or loss in that area. Several lines of evidence suggest that the modulation in the expression of WNT families was observed in the animal model of

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myocardial infarction, including the up regulation of WNT2, 4, 10b, and 11 as well as the down regulation of WNT7b [13]. In a cryoinjury model of neonatal mice, WNT3A, 4, 5b, 6, 8a, 9b, and 10b were upregulated in the myocardium of the injured hearts, and WNT2b, 5a, and 9a were upregulated in the injured epicardial layer [14]. Whereas WNT8b, 10a, 11, and 16 displayed similar expression in injured and uninjured hearts. Manipulation of WNT expression or secretion can produce beneficial or deleterious effects on infracted cardiac tissue. Overexpression of WNT10b or WNT11 in myocardial tissue led to a marked reduction in the inflammatory response in the infracted heart, an increase in neovascularisation, a smaller scar size and an improved ventricular function [15,16]. On the other hand, interruption with the lipidation of WNT ligands by the inhibitors GNF-6231 or WNT-974 to a mouse model of myocardial infarction also reduced infarct size, prevented pathologic remodelling, and reduced the decline in cardiac function [17,18]. Direct injection of recombinant WNT3A protein in the border zone of the infarct area after myocardial infarction in mice was shown to have detrimental effects on infarct healing, because this treatment blocked endogenous cardiac regeneration and impaired cardiac performance [19].

WNT3A is an important WNT ligand associated with cardiac development and cardiac diseases. It is reported that WNT3A favours mesoderm induction of embryonic stem cells through canonical signaling during cardiac differentiation [7]. Previous studies showed that WNT3A is triggered during the pathological process such as hypoxia and cell injury. In an *in vitro* model of neural ischemia and hypoxia, the expression levels of WNT1 and WNT3A proteins were significantly elevated [20]. However, the effect of WNT3A on cell survival varied among different cell types and condition. In pancreatic NIT-1 cell, WNT3A was found to promote proliferation and repress cytokine-induced beta-cell apoptosis [21]. WNT3A /β-catenin can also promote cell growth in heart valve interstitial cells [22]. A study found that WNT3A can inhibit serum starvation-induced apoptosis of HEK293 cells [23]. Moreover, overexpression of WNT3A has been shown to have protective effects on the survival of mouse embryonic liver stem cells and neuronal cells [24,25]. A study revealed that Bnip3 aggravated hypoxia induced cell injury by activating WNT/β-catenin signaling pathways in H9C2 cells [26]. WNT3A was found to augment cellular caspase activity in rat cardiomyoblasts subjected to hypoxia/reoxygenation treatment [27]. In contrast, another report suggested that the activation of WNT/β-catenin signaling by exosomes from adipose-derived mesenchymal stem cells could protect H9C2 cells against hypoxia/reoxygenation injury [28].

Despite controversial observations regarding the role of WNT3A, our results showed that overexpression of WNT3A aggravated OGD-induced cell injury in H9C2 cardiomyocytes. Mechanistic studies implicated that WNT3A deteriorated OGD-induced mitochondrial disorder and promoted cytosolic release of cytochrome C. Furthermore, we found that WNT3A overexpression directly reduced the expression of SIRT3.

SIRT3 is a member of the mammalian sirtuin families, which are homologs of the SIR2 protein found in yeast. In mammals, a total of seven sirtuin isoforms is found, Sirt1-7. Like SIRT1 and SIRT2, SIRT3 exhibits NAD+-dependent deacetylase activity. Nevertheless, they vary in subcellular localization and mechanisms of biological regulation. SIRT3 is mainly located in the mitochondrial matrix. Therefore, SIRT3 is a deacetylase that regulates the majority of mitochondrial lysine acetylation [10,29]. SIRT3 has been demonstrated to target a broad range of proteins involved in the oxidative stress, ischemia/reperfusion injury, mitochondrial metabolism homeostasis and cellular death. The expression of SIRT3 is high in the heart. Recent studies show that SIRT3 plays important roles in cardiovascular physiology and pathology. It was reported that SIRT3 level declined in the ischemia/reperfusion model induced by left anterior descending artery occlusion [30]. SIRT3 downregulation increases the susceptibility of adult hearts to ischemia/reperfusion injury. SIRT3 deficiency in mice caused coronary micro vascular dysfunction and impeded cardiac recovery post myocardial ischemia [31]. H9C2 cells silenced for SIRT3 expression were more susceptible to ischemia/reperfusion injury, as demonstrated by the augmented release of lactate dehydrogenase [32]. On the other hand, overexpression of SIRT3 could favour myocardial survival and reduce cardiac fibrosis in post-infarcted hearts [33]. In this study, we also observed that SIRT3 overexpression exerted protective effects on H9C2 cells. Simultaneously, SIRT3 overexpression was accompanied with WNT3A down regulation. Overall, emerging evidences implicated that SIRT3 exhibited a variety of cardio protective effects on ischemic heart disease. Herein, our study provided the new evidence that WNT3A can affect cardiomyocyte injury via down regulating SIRT3 expression. On the other hand, SIRT3 can also interrupt WNT3A expression. Acute MI decreased SIRT3 expression, while enhanced the level of WNT3A protein in a mouse model. Therefore, our findings implicate a complicated interaction between WNT3A and SIRT3 in myocardial ischemia.

Furthermore, our data showed that SFRP4 antagonized WNT3A and increased cell survival in H9C2 cells under the OGD condition. SFRPS (SFRPL-5) are a family of glycosylated WNT antagonists that structurally resemble Frizzled receptors but lack the transmembrane domain. Thus, SFRPS are thought to bind and sequester WNTs away from active receptor complexes. A solid phase binding assay demonstrated that SFRP2 physically binds to WNT3A [27]. SFRP1 and 2 dramatically attenuate WNT3A -induced accumulation and transcriptional activity of β-catenin, while SFRP3 cannot [34]. SFRP1 directly protected H9C2 cells from hypoxia/reoxygenation-induced reperfusion injury and apoptosis through inhibition of the WNT signaling pathway [35]. SFRP2 was characterized as a mesenchymal stem cell-released paracrine factor to improve myocardial survival and repair after ischemic injury [36]. Another study showed that SFRP4 was able to inhibit mesothelioma cell proliferation and migration via antagonizing WNT3A [11]. SFRP4 also contributes to the process of cardiac differentiation via cross talking with WNT, integrin, and Notch signals [37]. In this study, our data suggested that SFRP4 is also a protective factor in the OGD-induced injury. In contrast to WNT3A, SFRP4 administration up regulated the expression of SIRT3 and alleviated mitochondrial malfunction in the OGD condition.

In conclusion, our results indicate that WNT3A plays a pathophysiological role under hypoxia plus nutrient deprivation. Its antagonism by WNT antagonists such as SFRP4 may be beneficial for myocardial protection. Moreover, we identify SIRT3 as a crucial molecule regulated by WNT signalling. SIRT3 can protect myocardial cells from OGD-induced injury and down regulate WNT3A expression. Thus, the WNT3A -SIRT3 regulatory axis might be a promising target for the potential treatment of cardiac ischemia and hypoxia.

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