Resveratrol attenuates hypoxia/reoxygenation-induced Ca\(^{2+}\) overload by inhibiting the Wnt5a/Frizzled-2 pathway in rat H9c2 cells

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Abstract. Resveratrol is able to protect myocardial cells from ischemia/reperfusion-induced injury. However, the mechanism has yet to be fully elucidated. In the present study, it is reported that resveratrol has a critical role in the control of Ca\(^{2+}\) overload, which is the primary underlying cause of ischemia/reperfusion injury. Hypoxia/reoxygenation (H/R) treatment decreased the cell viability and increased the apoptosis of H9c2 cells, whereas the caspase-3 and intracellular Ca\(^{2+}\) levels were greatly elevated compared with the control group. Treatment of H9c2 cells with resveratrol (5, 15 and 30 µM) reduced caspase-3 expression and cardiomyocyte apoptosis in a dose-dependent manner, and the intracellular Ca\(^{2+}\) overload was also significantly decreased. Furthermore, Frizzled-2 and Wnt5a belong to the non-canonical Wnt/Ca\(^{2+}\) pathway, which have been demonstrated to be responsible for Ca\(^{2+}\) overload, and were thus detected in the present study. The results indicated that both the mRNA and protein expression levels of Frizzled-2 and Wnt5a in H/R-induced H9c2 cells were markedly increased compared with the levels found in normal cells, and treatment with resveratrol (5, 15 and 30 µM) significantly reduced the expression of Frizzled-2 and Wnt5a compared with the H/R group. The results indicated that resveratrol protected myocardial cells from H/R injury by inhibiting the Ca\(^{2+}\) overload through suppression of the Wnt5a/Frizzled-2 pathway.

Introduction

Acute myocardial infarction (AMI) is a common type of cardiovascular disease with high mortality and morbidity, and epidemiological evidence indicated that ~27% of 1 million patients have died of AMI in the US (1). One of the most significant factors responsible for the high mortality and poor recovery rate of AMI is the myocardial ischemia/reperfusion injury (MIRI), which occurs during revascularization therapy (2). MIRI leads to the apoptosis of a large number of cardiomyocytes by various mechanisms, including intracellular Ca\(^{2+}\) overload (3). Intracellular Ca\(^{2+}\) overload results in the opening of the mitochondrial permeability transition pores and depolarization of the inner mitochondrial membrane, all of which lead to cardiomyocyte apoptosis (4). Therefore, novel methods to prevent and attenuate the intracellular Ca\(^{2+}\) overload during MIRI are urgently required for the treatment of AMI.

Resveratrol is an edible polyphenolic phytoalexin found in grapes and red wine and has been identified to exhibit a wide range of biological and pharmacological properties, including anti-ageing, anti-inflammatory and anti-cancer effects (5-7). In addition, resveratrol has aroused attention due to its various cardioprotective effects (8). Studies have reported that resveratrol is able to attenuate ischemia-reperfusion injury, inhibit low-density lipoprotein oxidation and promote vasorelaxation, which clearly support its potential role for the prevention and therapy of MIRI (9,10). Additionally, Shen et al (11) identified that resveratrol alleviates the cardiomyocyte Ca\(^{2+}\) overload induced by H\(_2\)O\(_2\). However, the mechanisms underlying its effects against MIRI and Ca\(^{2+}\) overload are not well established.

The Wnt5a/Frizzled-2 signaling pathway is a non-canonical Wnt pathway responsible for the intracellular Ca\(^{2+}\) release (12). Upon binding to Wnt5a, the Frizzled-2 receptor induces Ca\(^{2+}\) release from intracellular stores and activates two kinases, Ca\(^{2+}\)/calmodulin-dependent protein kinase II and protein kinase C, in a G-protein dependent manner (13,14). It has been identified that Frizzled-2 overexpression activates the...
Wnt5a/FRizzled-2 pathway and thereby induces Ca\(^{2+}\) accumulation and cardiomyocyte apoptosis and that this effect can be reversed through the inhibition of Frizzled-2 (15). Therefore, resveratrol may protect cardiomyocytes from MIRI injury through the inhibition of Wnt5a/FRizzled-2.

In the present study, an H/R cardiomyocyte model was established in order to study the effects of resveratrol on the expression of Wnt5a/FRizzled-2 and intracellular Ca\(^{2+}\) overload. In addition, the cardiomyocyte activity and apoptosis were detected in order to determine the cardioprotection effects of resveratrol. The aim of the present study was to elucidate the mechanisms through which resveratrol protects cardiomyocytes in vitro.

Materials and methods

Hypoxia/reoxygenation model and experimental groups. H9c2 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle’s medium (DMEM) supplemented with 4.5 g/l glucose, 10% fetal bovine serum and 1% penicillin/streptomycin at 37\(^{\circ}\)C. The cardiomyocytes were incubated in normal Hank's solution throughout the experimental period; ii) the H/R group, in which the cardiomyocytes were incubated in normal Hank's solution at 37\(^{\circ}\)C without glucose or Ca\(^{2+}\) and then the cardiomyocytes were exposed to hypoxia by transferring the culture plates to a humidified incubation chamber thermoregulated at 37\(^{\circ}\)C with a gas mixture of 95% N\(_2\) and 5% CO\(_2\). Following 37 h, the cardiomyocytes were transferred to another chamber containing 95% air and 5% CO\(_2\) for reoxygenation. The cardiomyocytes were randomly divided into three groups as follows: i) the control group, in which the cardiomyocytes were incubated in normal Hank's solution throughout the experimental period; ii) the H/R group, in which the cardiomyocytes were treated under anoxic conditions for 3 h and under reoxygenation conditions for 3 h; and iii) the resveratrol group, in which the cardiomyocytes were treated as in the H/R group with the addition of resveratrol (Sigma Aldrich, St. Louis, MO, USA) at a final concentration of 5, 15 or 30 \(\mu\)M to the culture medium 10 min after reoxygenation. The cell viability and the lactate dehydrogenase (LDH) levels were detected at the end of the reoxygenation treatment, and all other determinations were performed following incubation of the cardiomyocytes with resveratrol for an additional 24 h (18).

Cell viability. A cell counting kit (CCK)-8 (Dojindo, Tokyo, Japan) was used to measure the cell viability according to the manufacturer's instructions. The cardiomyocytes were seeded in 96-well plates at 1\(\times\)10\(^4\) cells/well and pretreated with different concentrations of resveratrol (0, 5, 15 or 30 \(\mu\)M) for 48 h or treated with resveratrol 10 min after the initiation of reoxygenation. The cardiomyocytes were treated under anoxic conditions for 3 h and under reoxygenation conditions for 3 h. At the end of the treatment period, 10 \(\mu\)l WST-8 solution was added to the cardiomyocytes and the cells were incubated for 2 h at 37\(^{\circ}\)C. The absorbance of each well at 450 nm related to the reference absorbance at 630 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, 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) \(\times\) 100 (18).

Lactate dehydrogenase (LDH) activity. The membrane damage was monitored by measuring the LDH leakage. In total, 100 \(\mu\)l culture medium was added to assess the amount of LDH by measuring the levels of pyruvic acid with a spectrophotometer, (Shimadzu UV-2201, Shimadzu Corporation, Kyoto, Japan) and the absorbance was detected at a wavelength of 440 nm (15).

Flow cytometric analysis. The apoptotic cells were measured using a Fluorescein FragELMT DNA Fragmentation Detection kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the cardiomyocytes were collected 24 h after reoxygenation. Next, 100 \(\mu\)l binding buffer and 10 \(\mu\)l fluorescein isothiocyanate-labeled Annexin V (20 \(\mu\)g/ml) were added to each sample, and the samples were incubated in the dark for 30 min. After 5 \(\mu\)l propidium iodide was added, the cell apoptosis rate was analyzed using a FACScan flow cytometer (Becton-Dickinson, Franklin Lanes, NJ, USA).

Measurement of caspase-3 activity. The caspase-3 activity was evaluated using a commercialized caspase-3 assay kit (Biovision, Mountain View, CA, USA). In total, \(-1\times10^6\) cells were harvested, and the pellet was resuspended in lysis buffer. The protein levels were determined through the bicinchoninic acid assay according to the manufacturer's instructions. The caspase-3 activity was measured as the optical density. The absorbance at 405 nm of the released peptide nucleic acid (pNA) was monitored using a spectrophotometer.

Quantitative polymerase chain reaction (qPCR). The total RNA from H9c2 cells was isolated using a Small-Scale Phenol-Free Total RNA Isolation kit (Ambion, Austin, TX, USA). Reverse transcription was carried out in a volume of 20 \(\mu\)l containing 1 \(\mu\)g total RNA. The expression of Frizzled-2, Wnt5a and GAPDH was quantitatively checked in H9c2 cells using an ABI 7500 RT-PCR System (Applied Biosystems, Foster, CA, USA) as described previously (27). The Power SYBR Green PCR Master Mix (Lingke, Shanghai, China) was used as a double-stranded DNA-specific dye according to the manufacturer's instructions. The PCR conditions were 95\(^{\circ}\)C for 1 min, 40 cycles of 95\(^{\circ}\)C for 15 sec and 55\(^{\circ}\)C for 1 min for Frizzled-2, and 95\(^{\circ}\)C for 1 min, 40 cycles of 95\(^{\circ}\)C for 15 sec and 60\(^{\circ}\)C for 1 min for Wnt5a and GAPDH. The levels of Frizzled-2 and Wnt5a mRNA were calculated based on the 2\(-\Delta\Delta CT\) of the intervening and the control groups. The following primer sequences were used: GAPDH, forward 5'-AGG GCT GCC TTC TCT TGT GA-3' and reverse 5'-AAC TTG CCG TGG GTA GAG TCA-3'; Frizzled-2, forward 5'-TCG TTT TGC CCG TCT CT-3' and reverse 5'-TAG CGG AAT CGC TGC AT-3'; Wnt5a, forward 5'-CGG AGA TTG TGG ATC AGT TC-3' and reverse 5'-GTT CCC AGC TGC AAT TCT TG-3'. The primers were synthesized by the Guangzhou Land Biology Technology Company (Guangzhou, Guangdong, China).

Western blot analysis. The protein samples were prepared using the ProteoExtract Transmembrane Protein Extraction kit (Novagen, Merck KgaA, Darmstadt, Germany), and the protein
concentration was measured using a bicinchoninic acid protein assay kit (Bocai, Shanghai, China). Following blocking for 1 h in Tris-buffered saline and Tween-20 (TBST) containing 5% bovine serum albumin, the membranes were washed in TBST and probed with the following primary antibodies overnight at 4°C: Rabbit polyclonal antibody to Frizzled-2, rabbit polyclonal antibody to Wnt5a and goat polyclonal antibody to human GAPDH (Invitrogen, Carlsbad, CA, USA). The membranes were then incubated with goat anti-rabbit immunoglobulin G peroxidase-conjugated secondary antibody (Sigma Aldrich) and visualized by enhanced chemiluminescence according to the manufacturer's instructions. GAPDH was used as the internal loading control. For quantification, the Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA) was used to measure the integrated optical density (IOD) of the bands. The relative protein levels are expressed as the ratio to the levels of GAPDH.

Laser scanning confocal microscopy. The intracellular Ca$^{2+}$ was detected by laser scanning confocal microscopy (Carl-Zeiss, Jena, Germany). The cells were incubated with Fluo-3AM at 37°C for 60 min, and the fluorescence was monitored at 528 nm. Images (512x512 pixels) were acquired, and the quantitative analysis of the laser scanning confocal microscopy data was conducted using the Image-Pro Plus 6.0 software. The values are expressed as the IOD and represented as the mean ± standard error of the mean (SEM).

Statistical analysis. The data were analyzed using the SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA) and are expressed as the mean ± SEM. The statistical analysis was completed using one-way analysis of variance with Fisher's least-significant difference post-hoc tests for multiple comparisons of the means. Differences were considered to be statistically significant if P<0.05.

Results

Resveratrol improves cell activity and reduces LDH release. Initially, the cytotoxic effects of resveratrol on H9c2 cells were observed through the CCK-8 assay, and the results indicated that there were no significant differences in cell viability between the groups treated with different concentrations of resveratrol (Fig. 1B; P>0.05). Additionally, resveratrol increased the H/R cardiomyocyte viability in a dose-dependent manner (Fig. 1B; P<0.05). Furthermore, LDH was measured in order to determine the extent of cell injury, and the results revealed that resveratrol significantly decreased the LDH levels of H/R cardiomyocytes (Fig. 1A; P<0.05).

Effect of resveratrol on apoptosis of caspase-3 activity in H/R H9c2 cells. To investigate the cardioprotective effect of resveratrol on H/R cardiomyocytes, the apoptotic rate of cardiomyocytes was then examined. The cardiomyocytes were treated with resveratrol (5, 15 or 30 µM) 10 min after the beginning of reoxygenation, and the levels of apoptosis and caspase-3 were detected by flow cytometry 24 h after reoxygenation. The flow cytometry results revealed that the apoptotic rate in the H/R group was significantly elevated compared with that in the control group, and resveratrol treatment inhibited H/R-induced cardiomyocyte apoptosis (Fig. 2A and B). In order to further demonstrate the anti-apoptotic action of resveratrol, the caspase-3 activity was determined. Caspase-3 is a crucial mediator of programmed cell death (19). The caspase-3 activity was significantly increased in the H/R group compared with that in the control group. However, resveratrol treatment significantly reduced the caspase-3 activity in a concentration-dependent manner (Fig. 2C; P<0.05). Therefore, resveratrol may protect cardiomyocytes by reducing their apoptotic rate and inhibiting caspase-3 activity.

Resveratrol decreases intracellular Ca$^{2+}$ accumulation in H9c2 cells. Intracellular Ca$^{2+}$ accumulation is a primary cause of cardiomyocyte apoptosis in MIRI (20). To investigate the concrete mechanisms of cardiomyocyte apoptosis and to determine whether the anti-apoptotic action of resveratrol is associated with Ca$^{2+}$ overload, the effect of resveratrol on intracellular Ca$^{2+}$ accumulation was examined. The intracellular Ca$^{2+}$ levels of the H/R group were significantly increased compared with those observed in the control group (Fig. 3). Of note, resveratrol treatment (5, 15 or 30 µM) significantly decreased the intracellular Ca$^{2+}$ levels in a dose-dependent manner, similarly to the results observed in the analysis of the apoptotic rate and

![Figure 1. Effect of resveratrol on LDH activity and cardiomyocyte viability. (A) Resveratrol reduced the expression of LDH in H/R cardiomyocytes. *P<0.05 versus the control group; †P<0.05 versus the H/R group. (B) Resveratrol increased the cell viability of H/R cardiomyocytes as detected by CCK-8 in a dose-dependent manner. *P<0.05 versus the untreated group. The data are expressed as the mean ± standard deviation (n=6). LDH, lactate dehydrogenase; H/R, hypoxia/reoxygenation; CCK-8, cell counting kit.](image-url)
Figure 2. Effects of resveratrol on caspase-3 activity and apoptosis of H/R cardiomyocytes. (A) Cardiomyocytes were treated with resveratrol (5, 15, or 30 µM) 10 min after the start of reoxygenation, and the rate of apoptosis was detected by flow cytometry 24 h after reoxygenation. Resveratrol significantly decreased the apoptosis of H/R cardiomyocytes (P<0.05). (B) Quantified apoptotic rates of cardiomyocytes shown in A. (C) Caspase-3 activity was determined 24 h after reoxygenation, and resveratrol (5, 15 or 30 µM) inhibited caspase-3 activity. Values are expressed as the mean ± standard deviation (n=6); #P<0.05 versus the control group, *P<0.05 versus the H/R group. H/R, hypoxia/reoxygenation; FITC, fluorescein isothiocyanate; PI, propidium iodide; OD, optical density.

Figure 3. Effects of resveratrol on Ca\textsuperscript{2+} overload in H/R cardiomyocytes. (A) Intracellular Ca\textsuperscript{2+} was assessed by confocal microscopy 24 h after reoxygenation. Resveratrol (5, 15 or 30 µM) decreased intracellular Ca\textsuperscript{2+} in H/R cardiomyocytes in a dose-dependent manner. Magnification, x200. (B) Average intensity of intracellular Ca\textsuperscript{2+}. Values are expressed as the mean ± standard deviation of three independent experiments performed in triplicate. #P<0.05 versus the control group; *P<0.05 versus the H/R group. H/R, hypoxia/reoxygenation.
caspase-3 activity. Together, these results indicated that resveratrol protected cardiomyocytes from H/R injury and reduced apoptosis by inhibiting the intracellular Ca^{2+} levels.

**Effects of resveratrol on the gene and protein expression of Wnt5a and Frizzled-2.** It has been demonstrated that the Wnt5a/Frizzled-2 pathway regulates intracellular Ca^{2+} accumulation (12). Therefore, the possibility of resveratrol stimulation affecting intracellular Ca^{2+} accumulation via the Wnt5a/Frizzled-2 signaling pathway was examined. qPCR analysis revealed that the expression levels of Wnt5a and Frizzled-2 mRNA were significantly increased in H/R cardiomyocytes compared with the control group. However, resveratrol treatment (5, 15 or 30 µM) significantly reduced the levels of Wnt5a and Frizzled-2 mRNA in H9c2 cells (Fig. 4A). These results indicated that resveratrol inhibited Wnt5a and Frizzled-2 mRNA expression in a concentration-dependent manner. Furthermore, western blot analysis was performed in order to examine the Wnt5a and Frizzled-2 protein expression levels. Similar to the qPCR results, the western blot analysis revealed that the Wnt5a and Frizzled-2 protein levels were significantly lower in the resveratrol group compared with those in the H/R group (Fig. 4B). These results supported the hypothesis that resveratrol has a critical role in the inhibition of the Wnt5a/Frizzled-2 pathway, which has been proven to be responsible for intracellular Ca^{2+} accumulation. Therefore, resveratrol may reduce intracellular Ca^{2+} accumulation by inhibiting the Wnt5a/Frizzled-2 pathway.

**Discussion**

The present study revealed that resveratrol ameliorates the effects of Frizzled-2 mediated by the Wnt/Ca^{2+} pathway in H/R-induced cardiomyocytic Ca^{2+} overload and protects cardiomyocytes from injury and apoptosis. The Wnt5a/Frizzled-2 pathway has a critical role in Ca^{2+} overload, which is a major factor that induced cardiomyocyte apoptosis in AMI. Evidence was provided supporting the conclusion that resveratrol suppresses Ca^{2+} overload by downregulating the expression of the Wnt5a/Frizzled-2 pathway.

It has been shown that resveratrol has numerous functions, including cardioprotective effects during MIRI (21,22). In the present study, H9c2 cells were selected for the in vitro H/R cell model due to their wide applications in MIRI research (15,23). The levels of LDH and cell viability are used as indicators of cardiomyocyte injury. The cell viability was found to be decreased in cardiomyocytes undergoing H/R treatment and resveratrol treatment was identified to be capable of protecting against H/R-induced cell injury. Similarly, resveratrol treatment also decreased the LDH levels in H/R cardiomyocytes. Therefore, as previous studies have demonstrated, it was proven that resveratrol exerted a protective effect on H/R cardiomyocytes (18). It is believed that the cardioprotection induced by resveratrol is achieved through a preconditioning effect; however, it is difficult to restrict its administration as a pretreatment (24,25). Resveratrol was found to still function when applied after the initiation of reoxygenation, which
means that resveratrol exerted direct protective effects on H/R cardiomyocytes and may be used for the clinical treatment of MIRI.

It is widely recognized that reperfusion is a ‘double-edged sword’, as reperfusion itself may lead to massive cardiomyocyte apoptosis due to the I/R procedure (26). Consistent with previous studies, it was demonstrated that resveratrol significantly decreased the apoptotic rate of H/R-treated cardiomyocytes. The activity of caspase-3, which is a key factor of the caspase cascade, was also inhibited (27,28). However, there is controversy regarding the resveratrol dosage and its cardioprotective action; studies have reported that a low dosage of resveratrol resulted in anti-apoptotic effects, whereas a high dosage of resveratrol may transfer a death signal, enlarge the myocardial infarction size and promote cardiomyocyte apoptosis (29,30). The present study indicated that resveratrol is able to exert anti-apoptotic effects at a relatively low concentration (5-30 µM); however, the optimal dosage remains to be determined.

Ca\textsuperscript{2+} overload is the main pathological change of MIRI (31). The elevation in the intracellular Ca\textsuperscript{2+} levels directly triggers cell death, and an increase in the mitochondrial Ca\textsuperscript{2+} levels may induce depolarization of the inner mitochondrial membrane and the opening of mitochondrial permeability transition pores, which would give rise to adenosine triphosphate depletion and apoptosis (4,32-34). Thus, the control of the intracellular Ca\textsuperscript{2+} levels is a key factor in the treatment of reperfusion injury. A recent study demonstrated that resveratrol suppressed H\textsubscript{2}O\textsubscript{2}-induced Ca\textsuperscript{2+} inflow and indicated that resveratrol attenuated H/R-induced cell injury and apoptosis by inhibiting the Ca\textsuperscript{2+} overload (11). Consistent with the abovementioned study, Ca\textsuperscript{2+} release was demonstrated to be significantly decreased when H/R cardiomyocytes were treated with resveratrol. Additionally, resveratrol treatment also reduced the apoptosis of cardiomyocytes. Therefore, one plausible explanation is that resveratrol decreases H/R-induced cell injury by suppressing Ca\textsuperscript{2+} overload.

The mechanisms involved in Ca\textsuperscript{2+} overload require to be fully elucidated, and mitochondrial dysfunction, an increase in cell membrane permeability, and catecholamine are currently the primary known causes of Ca\textsuperscript{2+} overload (4,35,36). The Wnt5a/Frizzled-2 pathway, which is a well-known Ca\textsuperscript{2+}-modulating pathway, is activated upon inflammation or ischemia/anoxia stimulation (37,38). A previous study by our group proved that the Wnt5a/Frizzled-2 pathway has a vital role in Ca\textsuperscript{2+} release in H/R cardiomyocytes (15). To confirm whether resveratrol suppresses Ca\textsuperscript{2+} overload by inhibiting the Wnt5a/Frizzled-2 pathway, the expression levels of Wnt5a and Frizzled-2 were determined. The results revealed that the gene and protein levels of both Wnt5a and Frizzled-2 were elevated by the stimulation of H/R and that resveratrol downregulated their expression in a dose-dependent manner. Therefore, resveratrol may decrease Ca\textsuperscript{2+} overload due to H/R by inhibition of the Wnt/Frizzled-2 pathway.

In conclusion, the present study determined that resveratrol directly protected cardiomyocytes from H/R-induced reperfusion injury and apoptosis through inhibition of the Ca\textsuperscript{2+} via the Wnt5a/Frizzled-2 pathway in MIRI. However, the results only demonstrated the cardioprotective effects of resveratrol in vitro. Due to the complicated nature of the in vivo environment and the low bioavailability of resveratrol, further in vivo studies will be conducted in order to confirm the effects of resveratrol on Ca\textsuperscript{2+} overload and reperfusion injury.

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