Upregulation of connexin 43 and apoptosis-associated protein expression by high glucose in H9c2 cells was improved by resveratrol via the autophagy signaling pathway

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Abstract. The expression of connexin43 (Cx43) protein and the apoptotic rate of cardiomyocytes may be regulated by autophagy and associated with diabetic cardiomyopathy. It is possible that the beneficial effect of resveratrol on diabetic cardiomyocytes occurs via the autophagy pathway. However, it remains to be elucidated whether resveratrol treatment may attenuate the hyperglycemia-induced remodeling of Cx43 and apoptosis through the regulation of autophagy. H9c2 cardiac cells were incubated with 5.5 and 25 mM glucose, 25 mM glucose with chloroquine (50 μM), and 25 mM glucose with or without resveratrol (10, 25 μM) for 24 h. H9c2 cells were also incubated with 25 μM resveratrol in the presence of chloroquine (50 μM). Cell viability was determined using an MTT cell survival assay. Cytotoxicity was determined by quantification of the release of lactate dehydrogenase. The expression of Cx43, autophagic maker proteins [Beclin-1, p62 and microtubule-associated protein 1 light chain 3 (LC3)], apoptosis maker proteins (B-cell lymphoma-2 and Bcl-2 associated X protein), AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) were determined using western blotting. Resveratrol treatment led to reduced Cx43 expression levels compared with the 25 mM glucose treatment and significantly reduced the expression of apoptosis-associated proteins in H9c2 cells under hyperglycemic conditions. Autophagy was increased as indicated by the upregulation of Beclin-1 and p62 expression and the reduced LC3-II/LC3-I ratio. AMPK expression was increased, whereas mTOR expression was reduced in the resveratrol treatment groups. Treatment with chloroquine reversed effect of resveratrol. In conclusion, administration resveratrol may protect H9c2 cells against hyperglycemia-induced Cx43 upregulation and apoptosis, which may be mediated through the induction of the autophagy signaling pathway.

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Abbreviations: Chq, chloroquine; HG, high glucose; Cx43, connexin43; Res, resveratrol; AMPK; AMP-activated protein kinase; mTOR, mammalian target of rapamycin; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide

Key words: connexin43, H9c2 cells, autophagy, high glucose, resveratrol

Introduction

Apoptosis is involved in a series of cardiovascular ailments, including diabetic cardiomyocytes. Previous studies have identified a higher rate of apoptosis in diabetic hearts *in vivo* and *in vitro* (1,2). However, the underlying mechanisms of pathogenesis remain to be elucidated.

In order to effectively pump blood, the heart relies on gap junctions (GJ), which allow for the rapid propagation of the electrical impulse to all cardiomyocytes (3). Apart from the transmission of electrical signals, GJ also mediate the exchange of ions, nutrients and small molecules between adjacent cells (4). GJ consist of a transmembrane protein, termed connexin (Cx). Connexin 43 (Cx43) belongs to the Cx family and is the primary connexin in ventricular cardiomyocytes (5). Previous studies revealed that the abundance of Cx43 is altered during various cardiovascular diseases, such as arrhythmia (6), myocardial infarction (7), heart failure (8) and hypertension (9). Furthermore, previous studies suggested that Cx43 expression was also altered in hyperglycemic conditions (10-12). However, there is controversy on the expression of cardiac Cx43 in diabetic rats or cardiomyocytes exposed to hyperglycemic medium, which indicated that Cx43 may be strongly related with the dysfunction of the diabetic rat heart. Previous studies demonstrated that the autophagy/lysosome signaling pathway may be involved in the regulation of Cx43 protein degradation (8,13). Martins-Marques *et al* (14) revealed that ischemia-reperfusion induced degradation of cardiac Cx43 by autophagy involved the recruitment of Beclin-1 and p62.

Autophagy (also termed macroautophagy) is an intracellular bulk degradation process that involves the elimination of damaged or unused proteins and organelles such as the

mitochondria (15). Autophagy occurs on a regular basis in cells; however, dysfunction in its regulation may contribute to the pathology of various conditions, including ischemia-reperfusion (16), starvation (17), heart failure (8), hypertension (9) and diabetes (18,19), suggesting that autophagy may have an important role in heart disease. In addition, dysfunctional autophagy has been observed in the diabetic heart and associated with an increase in cardiac apoptosis, which was improved by the activation of autophagy following metformin administration (2). This is in accordance with a previous study which indicated that metformin could normalize cardiac autophagy and attenuate high glucose-induced apoptosis (1). Previous studies on induced autophagy reported that resveratrol treatment protected cardiac cells against injury under various pathological conditions, including diabetes, ischemia-reperfusion and myocardial infarction (20-22). However, excessive or restricted autophagy may lead to cell death in the heart. A previous study revealed that autophagy may have a detrimental effect in an alcoholic cardiomyopathy mouse model (23). Therefore, it remains to be elucidated whether autophagy is beneficial or detrimental to the heart. Previous studies determined that autophagic markers, including microtubule associated protein light chain 3 (LC3), Beclin-1 and p62 were required for cytoplasm-to-lysosome delivery (24-26).

Resveratrol is a natural polyphenol contained in wine, grapes and vegetables. This agent was investigated due to its potential health benefits associated with its cardioprotective, anti-inflammatory, antioxidant and antiplatelet properties (27-29). Previous studies suggested that resveratrol protected against apoptotic cell death and improved cardiac function in ischemia-reperfusion injury or post-infarction heart failure model via an autophagy-dependent pathway (21,22,27). In addition, a previous study demonstrated that resveratrol may be a potential therapeutic strategy for diabetic cardiomyopathy through the autophagy signaling pathway (20). To the best of our knowledge, this is the first study to investigate the effect of resveratrol on Cx43 expression and the role of autophagy in hyperglycemic conditions. The present study aimed to evaluate the effect of resveratrol on autophagy and Cx43 expression in H9c2 cardiac muscle cells exposed to a high glucose medium.

Materials and methods

Materials. Resveratrol and chloroquine were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). The H9c2 cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Lactate dehydrogenase (LDH) activity assay and MTT kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Primary antibodies against Cx43 (cat no. 3512), AMP-protein activated kinase (AMPK; cat no. 2532) and phosphorylated (p)-AMPK (cat no. 2531S), mammalian target of rapamycin (mTOR; cat no. 2972), p-mTOR (cat no. 2971), Beclin-1 (cat no. 3738), p62 (cat no. 5114), LC3-I/II (cat no. 2775), B-cell lymphoma-2 (Bcl-2; cat no. 2870) and Bcl-2-associated X protein (Bax; cat no. 2772) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture and treatments. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C in a humidified incubator with 5% CO_2. Confluent cells (80-90% confluence) were used for the subsequent experiments. The H9c2 cells were exposed to medium containing 5.5 mM glucose [control (Con) group], 25 mM glucose [high glucose (HG) group], or 25 mM glucose and resveratrol at concentration of 10 or 25 μM (Res10 and Res25 group, respectively) for 24 h. Additionally, H9c2 cells were incubated with 25 μM resveratrol in the presence of chloroquine (50 μM) in hyperglycemic conditions induced by 25 mM glucose.

Cell viability assay. Cell viability was quantified with an MTT assay. The H9c2 cells were seeded in 96-well plates at a density of $2.0x10^4$ cells/well. Following 24 h incubation, MTT solution (final concentration of 0.5 mg/ml) was added to each well and incubated for 4 h at 37˚C. Following the removal of the medium, DMSO was added to dissolve the blue-colored formazan product. Absorbance was measured with a microplate reader at 490 nm. Cell survival rate was expressed as the absorbance (optical density) of the various groups.

LDH release. Cell damage was assessed by the quantity of LDH recorded. The LDH assay kit was used according to the manufacturer's protocol. The cell medium was collected and then mixed with LDH reaction buffer for 30 min at room temperature. The reaction was stopped and the absorbance was measured at 490 nm using a microplate reader. Cell damage was expressed as the absorbance (optical density) of the various groups.

Western blotting. Following the treatments, H9c2 cells ($2.0x10^6$ cells/well) were harvested and lysed for 30 min at 4˚C with radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany). The supernatant fractions were collected by centrifugation at 10,000 x g for 20 min at 4˚C and protein concentration was determined using bicinchoninic acid assay kit (Beyotime Institute of Biotechnology). Equal amounts of extracted protein samples (10 μg) were separated by 12% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% (w/v) non-fat milk for 2 h at room temperature. The membranes were then incubated with the following rabbit polyclonal primary antibodies: Anti-Cx43 (1:1,000 dilution), anti-AMPK (1:1,000 dilution), anti-p-AMPK (1:1,000 dilution), anti-mTOR (1:1,000 dilution), anti-p-mTOR (1:1,000 dilution), anti-p62 (1:1,000 dilution), anti-Beclin-1 (1:1,000 dilution), anti-LC3 (1:1,000 dilution), anti-Bcl-2 (1:1,000 dilution), anti-Bax (1:1,000 dilution) and rabbit monoclonal anti-GAPDH antibody (cat no. 2118; 1:10,000 dilution; Cell Signaling Technology, Inc.) at 4˚C overnight. Subsequently, they were incubated for 2 h at room temperature with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat no. A0208; 1:4,000 dilution; Beyotime Institute of Biotechnology). Protein bands were visualized using enhanced chemiluminescence regent (EMD Millipore, Billerica, MA, USA) and were captured using the ImageQuant LAS 4000 (GE

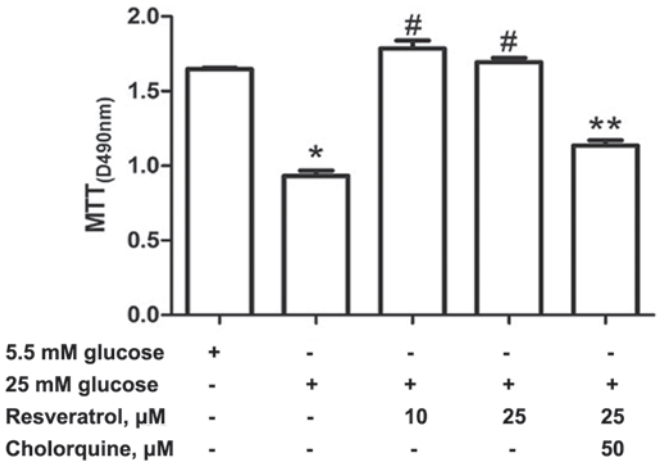

Figure 1. Resveratrol-mediated autophagy induced H9c2 cell viability. H9c2 cells were incubated in medium containing 5.5 and 25 mM glucose with different concentrations of resveratrol. Data are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. Con, #P<0.05 vs. HG, **P<0.05 vs. Res25. Con, control; HG, high glucose; Res25, resveratrol 25 μM.

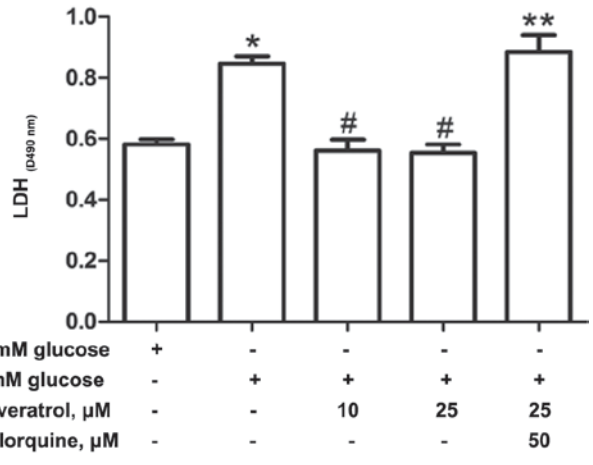

Figure 2. Resveratrol-mediated autophagy reduced H9c2 cell death, where LDH was used a marker of cell death. H9c2 cells were incubated in medium containing 5.5 and 25 mM glucose with different concentrations of resveratrol. Data are expressed as mean ± standard deviation (n=3). *P<0.05 vs. Con, #P<0.05 vs. HG, **P<0.05 vs. Res25. Con, control; HG, high glucose; Res25, resveratrol 25 μM; LDH lactate dehydrogenase.

Healthcare Life Sciences, Little Chalfont, UK) image reader. Blots were semi-quantified by densitometric analysis using Gel-Pro32 Analyzer software version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data were expressed as the mean ± standard deviation of 3 independent experiments. One-way analysis of variance followed by the Student-Newman-Keuls test was used to identify significant differences between HG and resveratrol groups. Student's t-test was performed to compare the differences between the Con and HG groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of resveratrol treatment on H9c2 cell viability. H9c2 cells were incubated with 5.5 or 25 mM glucose, or 25 mM glucose and resveratrol (10, 25 μM) for 24 h. The viability of H9c2 cells indicated by MTT assay was significantly increased in resveratrol group compared with the HG group (Fig. 1). However, treatment with 50 μM chloroquine and 25 μM resveratrol reduced cell survival compared with the 25 μM resveratrol group (Fig. 1).

Effect of resveratrol treatment on cell damage. Cell damage was indicated by LDH activity. The LDH activity was significantly higher in the HG group compared with the Con group (Fig. 2). However, H9c2 cells treated with resveratrol exhibited significantly reduced LDH activity compared with the HG group. Treatment with 50 μM chloroquine and 25 μM resveratrol significantly increased cell damage as LDH release was increased compared with the Res10 and Res25 groups.

Effect of resveratrol on Cx43 protein expression level in hyperglycemia-cultured H9c2 cells. Cx43 expression was quantified using western blot analysis (Fig. 3). Cx43 protein expression was significantly increased in the HG compared with the Con group (Fig. 3A and B). Notably, treatment with resveratrol prevented the hyperglycemia-induced increase in Cx43 protein expression levels (Fig. 3B). No significant difference was identified in terms of Cx43 expression between the resveratrol-treated groups and the Con group.

Effect of resveratrol on autophagy-associated proteins in hyperglycemia-cultured H9c2 cells. Following 24 h incubation with 25 mM glucose, the protein expression levels of autophagy-associated proteins were upregulated. In addition, treatment with resveratrol in hyperglycemia-cultured H9c2 cells induced autophagy. Autophagy is evaluated by the protein expression levels of LC3-II and p62. The p62 (Fig. 3D) expression level and the LC3-II/LC3-I ratio (Fig. 3E) were significantly increased in the HG group compared with the Con group, indicating the onset of autophagy. It is of note that p62 protein expression was further increased in the Res treatment groups compared with the HG group, whereas the LC3-II/LC3-I ratio was reduced in the Res-treated groups compared with the HG group. Furthermore, Beclin-1 protein expression levels were upregulated in the HG group and in the Res treatment groups compared with the HG group (Fig. 3C).

In the cells treated with resveratrol and chloroquine, which is an inhibitor of autophagy, the expression of p62 and the LC3-II/LC3-I ratio were further increased compared with the resveratrol only treatment groups, suggesting that resveratrol may increase autophagic turnover in H9c2 cells during hyperglycemic conditions. However, Beclin-1 expression was unaffected by chloroquine treatment (Fig. 3C).

Effect of chloroquine on resveratrol-induced Cx43 downregulation in hyperglycemia-cultured H9c2 cells. Resveratrol treatment was observed to increase autophagic flux in H9c2 cells under hyperglycemic conditions, along with downregulation of Cx43 expression. When the cells were incubated with 25 μM resveratrol and 50 μM chloroquine, Cx43 expression was increased compared with the 25 μM resveratrol alone treatment group, which indicated that resveratrol may promote

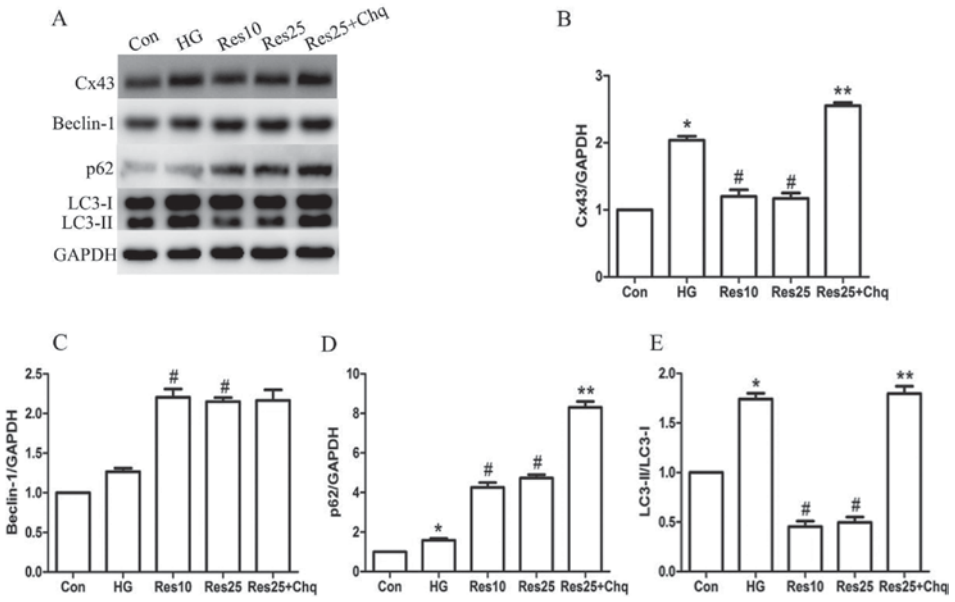

Figure 3. Resveratrol treatment prevented Cx43 upregulation through autophagy pathway. (A) Western blotting was performed in order to determine the expression of (B) Cx43, (C) Beclin-1, (D) p62 and (E) LC3-II/LC3-I. The target protein density was normalized to the control cells. Data are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. Con, $^{\#}$P<0.05 vs. HG, **P<0.05 vs. Res25. Cx43, connexin 43; LC3-I, -II, microtubule-associated protein 1 light chain 3-I, -II; Con, control; HG, high glucose; Res10, resveratrol 10 μM; Res25, resveratrol 25 μM; Chq, chloroquine.

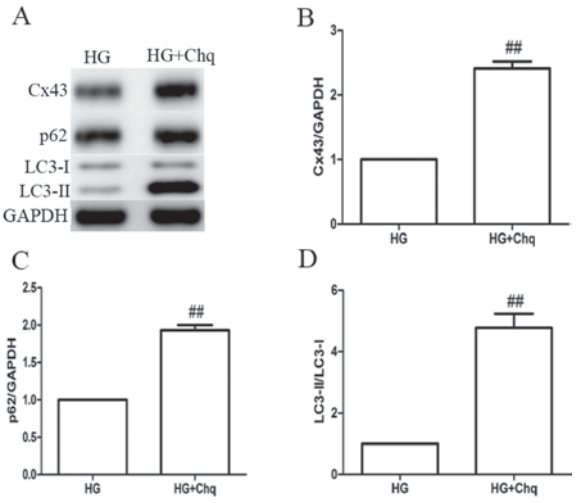

Figure 4. Chloroquine treatment increased Cx43 expression by reducing autophagic flux under hyperglycemic conditions. (A) Western blotting was performed in order to determine the expression of (B) Cx43, (C) p62 and (D) LC3-II/LC3-I. The target protein density was normalized to the control cells. Data are expressed as the mean ± standard deviation (n=3). $^{\#\#}$P<0.05 vs. HG. Cx43, connexin 43; LC3-I, -II, microtubule-associated protein 1 light chain 3-I, -II; HG, high glucose; Chq, chloroquine.

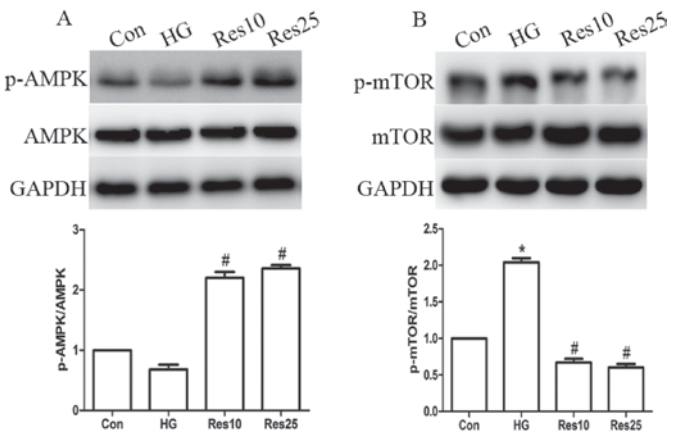

Figure 5. Resveratrol-mediated autophagy attenuated mTOR expression and activated AMPK. Western blotting to determine the expression levels of (A) AMPK and (B) mTOR. The target protein density was normalized to the control cells. Data are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. Con, $^{\#}$P<0.05 vs. HG. Con, control; HG, high glucose; Res10, resveratrol 10 μM; Res25, resveratrol 25 μM; AMPK, AMP-protein activted kinase; p-AMPK, phosphorylated-AMPK; mTOR, mammalian target of rapamycin; p-mTOR, phosphorylated-mTOR.

Cx43 downregulation through activation of the autophagy pathway (Fig. 4A and B).

Effect of chloroquine on Cx43 expression and autophagy activity in hyperglycemia-cultured H9c2 cells. Cells were incubated with chloroquine (50 μM) in hyperglycemic conditions and Cx43 expression was increased compared with the HG only group. The p62 expression level and LC3-II/LC3-I ratio were also increased compared with the HG only group, suggesting that the inhibition of autophagy was prevented in the chloroquine group (Fig. 4C and D). Beclin-1 expression was unaffected by chloroquine treatment (data not shown).

Resveratrol treatment upregulates AMPK and downregulates mTOR expression levels. It has been previously established that AMPK may act as an energy sensor, which is activated in response to a reduction in ATP content (22). The present study revealed a reduction in the phosphorylation level of AMPK in the HG group, which represents the activated form. However, resveratrol treatment increased the level of p-AMPK without affecting the total AMPK (Fig. 5A). In addition, it was determined that the phosphorylation and activation of mTOR

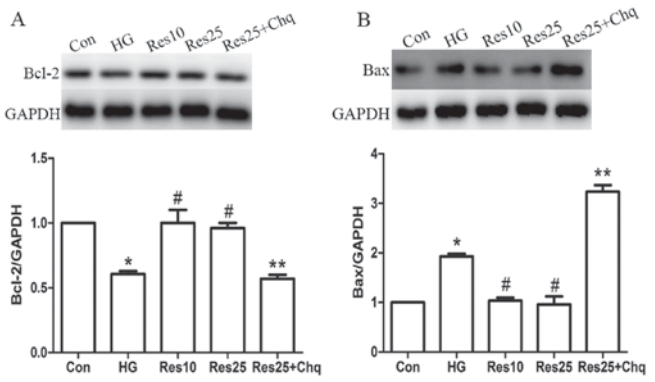

Figure 6. Resveratrol treatment attenuated the expression levlels of apoptosis-associated proteins. Western blotting was performed to determine the expression levles of (A) Bcl-2 and (B) Bax. The target protein density was normalized to the control cells. Data are expressed as the mean ± standard deviation (n=3). *P<0.05 vs. Con, #P<0.05 vs. HG, **P<0.05 vs. Res25. Con, control; HG, high glucose; Res10, resveratrol 10 μM; Res25, resveratrol 25 μM; Chq, chloroquine; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

(p-mTOR) was increased in the HG group. However, resveratrol treatment increased the expression of p-mTOR without affecting total mTOR expression (Fig. 5B).

Resveratrol ameliorates cell apoptosis depending on autophagic induction. Bcl-2 and Bax protein expression levels were used to assess apoptosis. Resveratrol treatment increased Bcl-2 expression and reduced Bax expression compared with the HG group. However, when the autophagy flux was inhibited by chloroquine, the effect of resveratrol was abolished as Bcl-2 expression was reduced and Bax levels were increased compared with the Res25 group (Fig. 6).

Discussion

The primary findings of the present study included the inhibitory effect of resveratrol on hyperglycemia-induced Cx43 upregulation in H9c2 cells combined with an increase in autophagic flux and resveratrol treatment led to an increase in autophagic flux along with increased cell survival and reduced cell damage in H9c2 cells exposed to hyperglycemic conditions.

Gap junctions primarily consist of Cx43 in ventricular cardiomyocytes, which have an important role in the maintenance of normal heart functions, such as orderly electric and metabolic coupling (3). Therefore, changes in the quantity of Cx43 expression has been associated with various heart diseases, such as myocardial infarction, ischemia-reperfusion, arrhythmia, diabetes and heart failure (7,8,10,12,30-32). A previous study demonstrated that Cx43 expression was significantly reduced in ischemic conditions, thus contributing to the impairment of impulse propagation (33). Greener *et al* (33) demonstrated that transfection with Cx43 could improve conduction velocity and reduce ventricular arrhythmia susceptibility in pigs with myocardial infarction. This was also observed by Rutledge *et al* (7), when mice were treated with c-Src inhibitors following a myocardial infarction. However, the alterations of Cx43 expression in diabetic hearts have remained controversial. It was previously reported that Cx43 expression was markedly reduced in various tissues under hyperglycemic conditions (34,35). However, Howarth *et al* (12) reported that total Cx43 expression was increased in hearts isolated from 12 weeks-old diabetic rats, and prolonged QT interval and QRS complex were observed. Therefore, it is of note, that increased cardiac Cx43 expression may not be consistently beneficial for the heart under certain circumstances. These findings were supported by Kanno *et al* (36).

In the present study, the expression of Cx43 increased significantly in H9c2 cells in hyperglycemic conditions, whereas resveratrol protected H9c2 cells from the increase in Cx43 induced by hyperglycemia. Therefore, it is possible that reduced Cx43 expression following resveratrol treatment may have a beneficial effect under the experimental conditions used in the present study.

Previous studies determined that Cx43 expression levels may be regulated by the autophagy signaling pathway (3,8,14). Autophagy may provide a source of energy when nutrients are scarce and may also be involved in protein quality control through discards defective cytoplasmic components. Previous studies have demonstrated autophagy-mediated the degradation of Cx43 *in vivo* and *in vitro* (14). The present study revealed that resveratrol treatment may lead to degradation of Cx43 in H9c2 cells through activation of the autophagy signaling pathway. Therefore, multiple autophagy-associated proteins including Beclin-1, p62 and LC3 were investigated. The current study determined that the levels of Beclin-1 and p62 were markedly increased, whereas the LC3-II/LC3-I ratio was significantly reduced in the resveratrol treatment group. The accumulation of p62 may indicate either an increase in autophagosome formation or a reduction in autophagic clearance (22). Therefore, to examine autophagic flux *in vitro*, the present study used chloroquine in combination with resveratrol. It has been previously established that chloroquine may inhibit lysosome fusion with autophagosomes and elevate lysosomal pH, thus preventing the final digestion step and inhibiting lysosomal activity, and is widely used as an autophagic inhibitor (22). Therefore, the LC3-II/LC3-I ratio and p62 expression were increased in H9c2 cells treated with chloroquine and resveratrol, and Cx43 expression was also upregulated. However, Beclin-1 protein expression was not affected by the chloroquine and resveratrol treatment. These findings suggested that resveratrol treatment increased autophagic flux as opposed to reducing it, and the downregulation of Cx43 expression by resveratrol treatment occurred via the activation of the autophagy pathway. It is of note that the present study also observed that the LC3-II/LC3-I ratio and p62 expression were increased following chloroquine treatment under hyperglycemic conditions. Furthermore, a hyperglycemia-induced increase in Cx43 expression was observed in the HG group, which may occur through an alternative process, such as increased synthesis, whereas the net effect of the HG treatment led to increased Cx43 expression. However, further investigation is required to determine the underlying mechanism.

In the present study, the expression levels of p62 were increased and the LC3-II/LC3-I ratio was reduced in the resveratrol treatment groups, which was inconsistent with

previous studies (21,22). Previous studies demonstrated that resveratrol increased autophagic flux, as indicated by increased LC3-II/LC3-I ratio and reduced p62 expression (21,22). Guidelines for monitoring autophagy suggest that if the degradation process of LC3-II due to lysosomal activity is rapid, it would it would lead to a decrease in LC3-II/LC3-I ratio (26). In addition, changes to LC3 may be rapid, whereas clearance of substrates of autophagy may require more time. Furthermore, p62 upregulation has also been observed in conditions where there is an increase in autophagic flux (37,38). Therefore, combined with increased Beclin-1 protein expression and the findings from the chloroquine treatment, the present study determined that induction of autophagic flux by resveratrol treatment is possible under the given set of experimental conditions.

In order to investigate the mechanisms of resveratrol-induced autophagic flux, the present study examined mTOR expression in hyperglycemic conditions. A previous study revealed that inhibition of mTOR by dephosphorylation may induce autophagy (21). It has been revealed that inhibition of mTOR with rapamycin induced autophagy in H9c2 cells (21). In the present study, resveratrol treatment inhibited mTOR dephosphorylation at serine 2448, along with an increase autophagic flux. These findings were consistent with a previous study (21), where resveratrol increased autophagy activity via inhibition of the phosphorylation of mTOR (Ser2448). However, high glucose treatment in the present study increased the activation of mTOR by enhancing the phosphorylation level at Ser2448, the autophagic flux was also increased. It is possible that hyperglycemia-induced autophagy is independent from inhibition of mTOR under hyperglycemic conditions. These findings suggested that resveratrol treatment increased the activation of autophagy primarily through preventing the phosphorylation of mTOR under the experimental conditions of the present study.

AMPK may act as a regulator of energy balance, and has several cellular functions including autophagy in the cardiovascular system (21,22). A previous study revealed that AMPK triggered autophagy through negatively regulating mTOR activity in H9c2 cells treated with resveratrol (21). The present study observed reduced AMPK expression in H9c2 cells under hyperglycemic conditions, whereas resveratrol treatment increased AMPK activation indicated by the increased phosphorylation level at Threonine 172. Therefore, it is possible that resveratrol-activated AMPK stimulated autophagic activity in H9c2 cells by modulating the mTOR pathway.

It has been previously reported that autophagy may regulate cell survival and cell death. He *et al* (1) demonstrated that induction of autophagy protected cells against apoptosis *in vivo* and *in vitro*. However, inhibition of autophagy may suppress this effect. Matsui *et al* (16) revealed that inhibition of autophagy in mice exposed to ischemia/reperfusion leads to reduced apoptotic cell death. The present study determined that resveratrol promoted cell survival, and Bcl-2 expression was increased whereas Bax expression was reduced. In addition, MTT assay and LDH release experiments also confirmed these observations. This effect was inhibited by chloroquine treatment, suggesting that autophagy is beneficial under hypoglycemic conditions.

Resveratrol was not observed to have a dose-dependent effect on the parameters examined in the current study. Gurusamy *et al* (21) demonstrated that 0.1 μM resveratrol was sufficient to induce autophagy in H9c2 cells. Therefore, the two concentrations of resveratrol (10 and 25 μM) used in the present study may have had a similar effect on H9c2 cells.

In conclusion, the findings of the present study revealed that resveratrol treatment led to downregulation of Cx43 expression and contributed to cell survival in H9c2 cells via regulation of autophagic flux. This is supported by the finding that inhibition of autophagic flux protected against hyperglycemia-induced Cx43 upregulation and apoptosis. The findings of the current study demonstrated that the AMPK/mTOR-mediated autophagy pathway may be involved in this condition. Further investigation is required to elucidate the underlying mechanism behind the resveratrol-induced autophagy in hyperglycemic conditions.

There are several limitations to the present study. Although chloroquine is a widely-used inhibitor of autophagy, this pharmacological approach lacks specificity. Therefore, a genetic approach may be more effective. Electron microscopy is the most accurate method for measuring autophagy; however, the present study was only able to perform western blotting for autophagy-associated proteins. Future investigations should aim to quantify the autolysosome via electron microscopy to clarify the effect of resveratrol on autophagy. Autophagy is a dynamic process; therefore, it will be useful for the effects of the treatments to be quantified at multiple time points. The findings of the present study should be replicated in other cell types and animal models, which may further elucidate the underlying mechanisms.

Acknowledgements

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