Upregulation of connexin43 by glucose deprivation in H9c2 cells via the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway

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Abstract. Cardiac connexin43 (Cx43) serves an essential role in maintaining the functional integrity of the heart. The present study investigated the effect of glucose deprivation (GD) on Cx43 protein expression levels in H9c2 cells, and demonstrated that following 2 h GD, Cx43 protein expression levels in H9c2 cells increased by ~68%. In addition, GD activated the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling pathway, which regulated the expression levels of cardiac Cx43. A MAPK inhibitor and U0126, an ERK inhibitor, abolished the effects of GD on Cx43 expression levels. Under GD, the protein expression levels of Beclin-1, p62 and LC3 were augmented, and were decreased in the presence of ERK inhibitor or siRNA-ERK. In addition, H9c2 cells exposed to GD exhibited marked increase in LDH release and decreased MTT reduction activity, all of which were not significantly reversed by U0126 treatment. Therefore, the ERK/MAPK signaling pathway may be involved in elevating cardiac Cx43 expression levels under GD in H9c2 cells.

Introduction

Cardiac gap junctions serve a crucial role in maintaining heart tissue homeostasis. There is abundant evidence that alterations of gap junctional intercellular communication (GJIC) is strongly associated with the development of heart diseases, including hypertension (1), arrhythmias (2,3), atherosclerosis and restenosis (4). It has been demonstrated that connexin43 (Cx43) is predominantly located in ventricular muscles (5). Cx43 forms gap junction channels and is additionally involved in the modification of cell-cycle (6). Accumulating evidence has indicated that Cx43 expression levels decrease in diabetes (7,8), myocardial infraction (2,3) and heart failure (9). In heterozygous Cx43-deficient mice, reduced Cx43 expression levels greatly increased the risk of ventricular arrhythmias (10). Downregulation of Cx43 may activate endothelial cells to a pathological status (11). Taken together, these results suggest that the reduction of cardiac Cx43 expression levels is a potential indicator of heart dysfunction.

Hypoglycemia is the most common side effect of exogenous insulin or insulin secretagogue administration in patients with type 1 and 2 diabetes. Hypoglycemia has been demonstrated to be strongly associated with adverse cardiovascular events and mortality (12). Numerous efforts have been made to identify potential underlying mechanisms between hypoglycemia and cardiovascular events. An increase in sympathetic system activity may lead to destabilization of atherosclerotic plaques during hypoglycemic episodes (13). In addition, previous studies have indicated that hypoglycemic episodes may induce cardiac and cerebral ischemia (14), arrhythmia (15), thrombosis and inflammation (16), all of which are strongly associated with the pathogenesis of cardiovascular diseases. However, establishing a direct causal link is difficult. Cardiac Cx43 acts as a potential biomarker of heart function; however, the association between cardiac Cx43 and hypoglycemia remains unclear.

Based on previous observations, the present study aimed to investigate the effect of glucose deprivation (GD) on Cx43 expression levels in H9c2 cells and to examine the underlying mechanisms.

Materials and methods

Cell culture and reagents. H9c2 cells were obtained from the Cell Bank of Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Waltham, MA, USA) at 37˚C in a humidified incubator in 5% CO_2 and 95% air.

U0126 and diphenyleneiodonium (DAPI) were purchased from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany).

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Abbreviations: Cx43, connexin43; HG, high glucose; NG, no glucose; GD, glucose deprivation; siRNA, small interfering RNA; LDH, lactate dehydrogenase

Anti-extracellular signal-regulated kinase (ERK; cat no. 9102; dilution, 1:1,000), anti-phosphorylated (p)-ERK (cat no. 9101; dilution, 1:1,000), anti-Cx43 (cat no. 3512; dilution, 1:1,000), anti-Beclin-1 (cat no. 3738; dilution, 1:1,000), anti-p62 (cat no. 5114; dilution, 1:1,000), anti-microtubule-associated protein 1A/1B-light chain 3 (LC3; cat no. 2775; dilution, 1:1,000), anti-B-cell lymphoma 2 (Bcl-2; cat no. 2870; dilution, 1:1,000) and anti-Bcl-2-associated X protein (Bax; cat no. 2772; dilution, 1:1,000) primary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA).

GD treatment. H9c2 cells were exposed to media containing no glucose (NG) for 2 h. The U0126 group were treated with 15 μM U0126 1 h prior to and during GD. The high glucose (HG) group was exposed to medium containing HG for 2 h.

Cell viability assay. Cell viability was measured by MTT assay. H9c2 cells were seeded into 96-well plates at a density of $2x10^4$ cells/well. After 24 h culture, the cells were subjected to GD treatment in the presence or absence of U0126. MTT solution (0.5 mg/ml; Beyotime Institute of Biotechnology, Shanghai, China) was added to each well and incubated for 4 h at 37˚C. Following this, the medium was removed and dimethyl sulfoxide was added to dissolve the blue-colored formazan product. Absorbance was measured at a wavelength of 490 nm using a microplate reader. Cell survival rates were expressed as the percentage of the absorbance of treated cells compared with the sham group.

Lactate dehydrogenase (LDH) release. Cell death was assessed using an LDH Activity Assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Cell medium was collected and mixed with LDH reaction buffer for 30 min at room temperature. Following this, the absorbance was read at a wavelength of 450 nm using a microplate reader.

Transfection of small interfering RNA (siRNA). siRNA-ERK was synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Primer sequences were as follows: Forward, 5'-GCU AUUACCGAGCAGACUUTT-3' and reverse, 5'-AAGUCU GCUCGGUAAUAGCTT-3' for ERK. H9c2 cells were seeded into 6-well plates to ensure 50-60% confluence and subsequently transfected with siRNA. Briefly, 1 μl siRNA-ERK was mixed with 3 μl transfection reagent, diluted with 250 μl transfection buffer (both from Qiagen GmbH, Hilden, Germany) and subsequently incubated for 25 min at 37˚C. Cells were incubated with the media for 24 h at 37˚C, following which the medium was replaced and cells were incubated for a further 48 h. The efficacy of transfection was measured by western blot analysis.

Immunofluorescence. Confluent cells were seeded onto coverslips and fixed with 4% paraformaldehyde. Following fixation, 0.1% Triton X-100 and 10% goat serum were used to permeabilize cells and saturate the non-specific binding sites, respectively. Cells were subsequently incubated with anti-Cx43 (4˚C overnight), followed by incubation with an Alexa Fluor 568 goat secondary antibody (cat no. 175471; Abcam, Cambridge, MA, USA; 25˚C for 2 h; dilution, 1:2,000). Finally, cell nuclei were stained with 0.5 μg/ml DAPI. The cells were examined using an inverted fluorescent microscope.

Western blot analysis. Following treatment, H9c2 cells were harvested and lysed 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 Applied Science, Penzburg, Germany). The supernatant fractions were collected by centrifugation (10,000 x g for 15 min at 4˚C) and protein concentration was determined using a Bicinchoninic Acid kit (Beyotime Institute of Biotechnology). Lysate protein was separated by 10-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies against ERK, p-ERK, Cx43, Beclin-1, p62, LC3, Bcl-2 and Bax at 4˚C overnight. Subsequently incubated for 2 h with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature. Proteins were detected using Enhanced Chemiluminescence reagents (EMD Millipore, Billerica, MA, USA). The bands were imaged using the LAS-4500 system and subsequently quantified using Gel-Pro Analyzer software (version 4.0; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Data are expressed as mean ± standard deviation. Student's t-test was performed to compare the differences between the HG and NG groups, and between NG and U0126 or siRNA groups. Statistical analysis was performed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability and LDH release. Compared with the HG group, cell survival rates significantly decreased and LDH release markedly increased during GD for 2 h in the NG group ($P < 0.05$). Compared with the NG group, U0126 administration had no effect on cell viability (Fig. 1A) and LDH release (Fig. 1B; $P > 0.05$).

Cx43 expression levels. Cx43 protein expression levels were significantly elevated in H9c2 cells exposed to GD ($P < 0.05$; Fig. 2A). Notably, the effect of GD on Cx43 expression levels in H9c2 cells was attenuated by U0126 treatment ($P < 0.05$; Fig. 2A), whereas U0126 treatment alone demonstrated no significant effect on Cx43 expression levels without GD (data not shown).

Activation of the ERK/MAPK signaling pathway. GD treatment activated the phosphorylation levels of ERK, as measured by western blotting ($P < 0.05$), whereas total ERK expression levels remained unaltered. As presented in Fig. 2B, the ERK inhibitor totally abolished GD-induced Cx43 upregulation. These results suggested that the ERK/mitogen-activated protein kinase (MAPK) signaling pathway may be involved in the effect of GD on Cx43 expression levels.
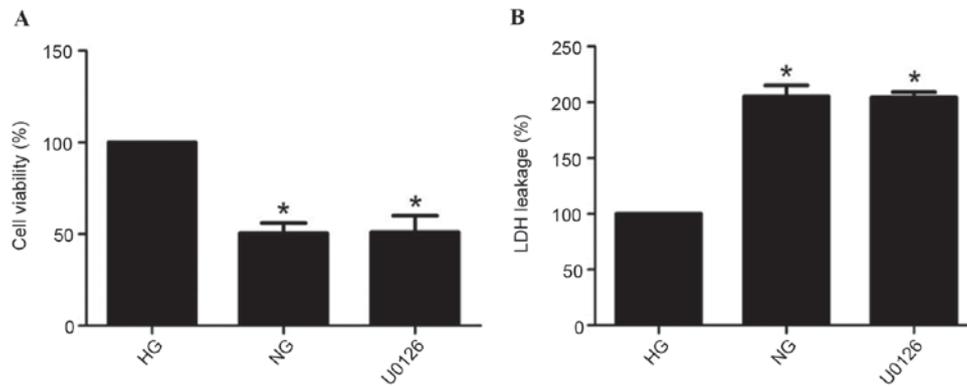
Figure 1. The effect of glucose deprivation on H9c2 cell survival. (A) Compared with the HG group, cell viability was significantly decreased in the NG group. No significant differences were observed between the NG and U0126 groups. (B) LDH release was significantly increased or remained unaltered in the NG group compared with the HG and U0126 groups, respectively. Data are expressed as the mean ± standard deviation. *P<0.05 vs. HG group. HG, high glucose; NG, no glucose; LDH, lactate dehydrogenase.
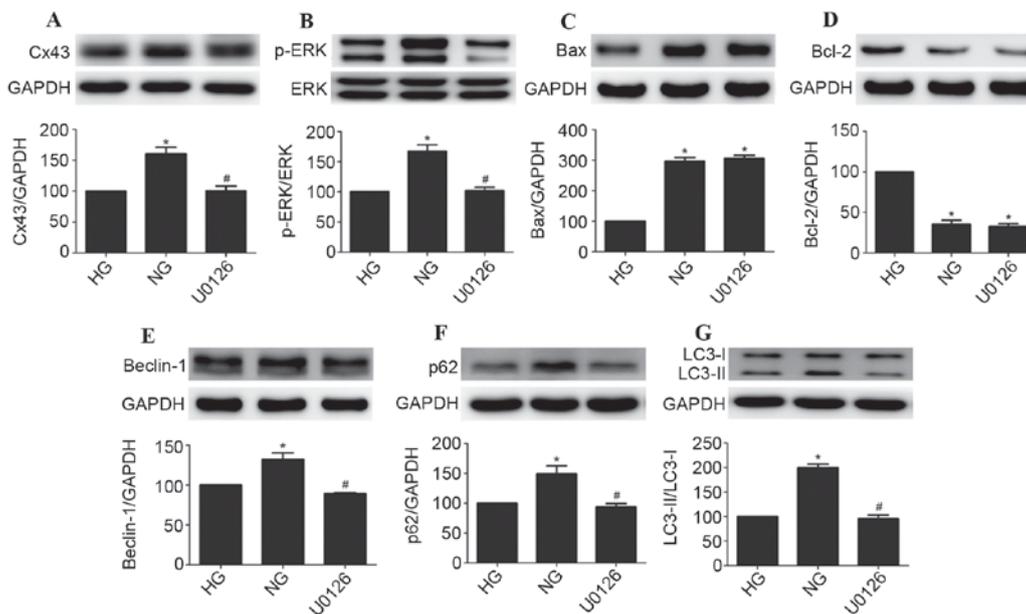

Figure 2. Effect of GD on Cx43, ERK, p-ERK, Bax, Bcl-2 protein expression and autophagy indicator levels in H9c2 cells. (A) Compared with the HG group, Cx43 expression levels were significantly increased in the NG group. U0126 (ERK inhibitor) treatment abolished the effect of GD on Cx43 expression levels. (B) Compared with the HG group, p-ERK expression levels were significantly increased in the NG group; however, p-ERK protein expression levels were inhibited following U0126 treatment under GD conditions. Total ERK expression levels remained unaltered. Compared with the HG group, (C) Bax expression levels were significantly increased and (D) Bcl-2 expression levels were markedly decreased in the NG group. U0126 treatment marginally altered these protein expression levels; however, not significantly. (E-G) Compared with HG group, (E) Beclin-1, (F) p62 and (G) LC3 expression levels were significantly increased in the NG group, and U0126 treatment abolished the effect of GD on these protein expression levels. Data are expressed as the mean ± standard deviation. *P<0.05 vs. HG group; $^#$P<0.05 vs. NG group. GD, glucose deprivation; Cx43, connexin43; ERK, extracellular signal-regulated kinase; p-, phosphorylated; Bax, B-cell lymphoma-2-associated X protein; Bcl-2, B-cell lymphoma 2; HG, high glucose; NG, no glucose; LC3, microtubule-associated protein 1A/1B-light chain 3.

Cell apoptosis. Compared with the HG group, protein expression levels of Bax were increased (Fig. 2C), whereas Bcl-2 protein expression levels were decreased (Fig. 2D) in GD-stimulated cells (P<0.05). However, no significant differences were observed in Bax and Bcl-2 expression levels between the NG and U0126 groups.

Autophagy-associated protein expression levels. Protein expression levels of Beclin-1 (Fig. 2E), p62 (Fig. 2F) and LC3 (Fig. 2G) were significantly increased in the NG group compared with the HG group (P<0.05). However,

inhibition of the ERK/MAPK signaling pathway (U0126 treatment) completely abolished the effect of GD on the protein expression levels of Beclin-1, p62 and LC3 (P<0.05).

Immunofluorescence of the effect of GD on Cx43 expression and localization. Consistent with the western blotting results, immunofluorescence microscopy of HG group cells demonstrated reduced levels of Cx43 localizing to cell borders in H9c2 cells (Fig. 3A). Increased immunoreactivity of Cx43 was observed between adjacent cells following GD
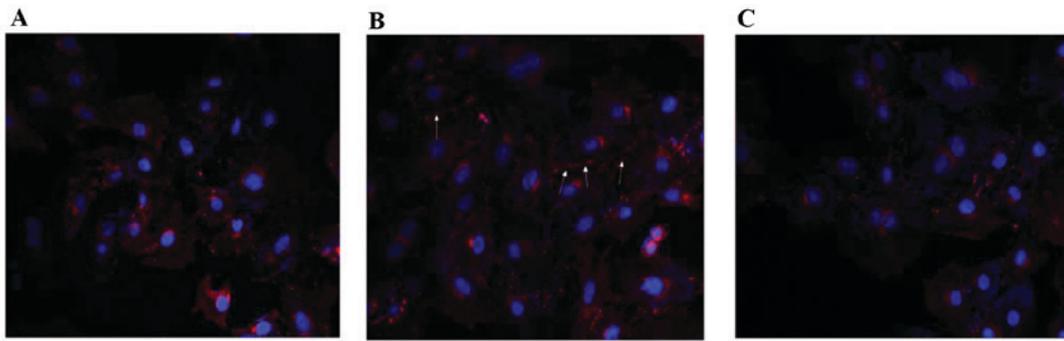
Figure 3. Effect of GD on Cx43 expression levels and localization. (A) In the HG group, levels of Cx43 were reduced at cell borders. Immunoreactivity signals were present in the cytoplasm. (B) In the no glucose group, Cx43 expression levels increased at cell borders, as indicated by the white arrows. (C) Cx43 expression levels decreased following 15 μM U0126 treatment compared with non-treated GD cells. Magnification, x200. GD, glucose deprivation; Cx43, connexin43; HG, high glucose.
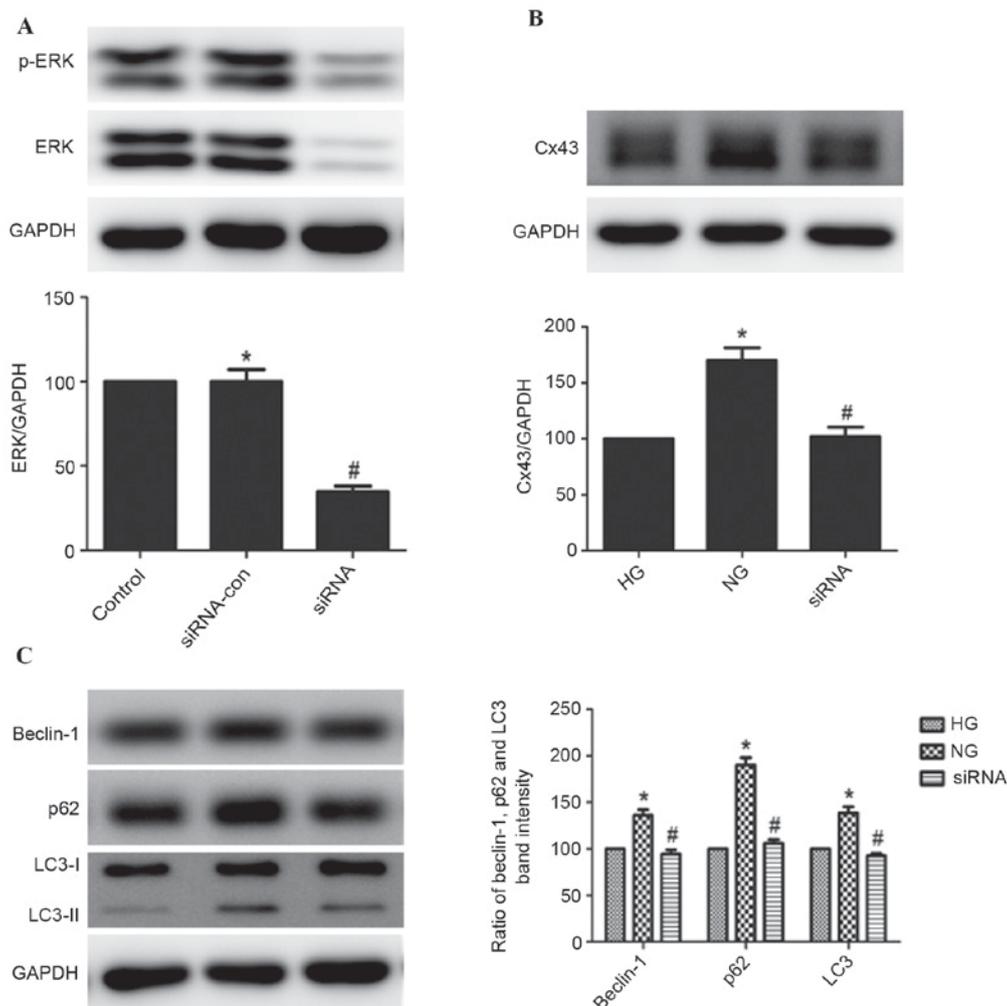

Figure 4. Effect of ERK-specific siRNA on Cx43 expression and autophagy indicator levels in H9c2 cells under GD conditions. (A) Compared with the control and siRNA-con groups, total ERK and p-ERK protein expression levels were significantly reduced in the siRNA group. (B) Compared with the HG group, Cx43 expression levels were significantly increased in the NG group; however, Cx43 protein expression levels were inhibited by siRNA-ERK treatment under GD conditions. (C) Compared with the HG group, Beclin-1, p62 and LC3 protein expression levels were significantly increased in the NG group. siRNA-ERK abolished the effect of GD on the expression levels of these proteins. Data are expressed as the mean ± standard deviation. *$P<0.05$ vs. HG group; #$P<0.05$ vs. NG group. ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; Cx43, connexin43; GD, glucose deprivation; p-, phosphorylated; HG, high glucose; NG, no glucose; LC3, microtubule-associated protein 1A/1B-light chain 3; siRNA-con, non-silencing ERK.

for 2 h (Fig. 3B). U0126 administration significantly decreased levels of Cx43 compared with GD treatment (Fig. 3C).

Silencing of the ERK/MAPK signaling pathway. As presented in Fig. 4A, total ERK and p-ERK protein expression levels

were inhibited by siRNA-ERK (P<0.05). Furthermore, Cx43 expression levels were significantly decreased in H9c2 cells transfected with siRNA-ERK under GD conditions (P<0.05; Fig. 4B). Protein expression levels of Beclin-1, p62 and LC3 were markedly decreased in the siRNA group compared with the NG group (P<0.05; Fig. 4C).

Discussion

The present study demonstrated that Cx43 protein expression levels markedly increased under GD conditions, accompanied by an increase in p-ERK expression levels. Immunocytochemistry indicated that increased Cx43 localization at the membrane between neighboring cells, consistent with the results of the western blot analysis. Furthermore, Cx43 expression levels in H9c2 cells under GD conditions were decreased following inhibition of p-ERK, indicating that p-ERK may be involved in the regulation of cardiac Cx43 expression levels during GD. Similarly, decreased Cx43 expression levels were observed by silencing ERK under GD conditions. Notably, upregulation of Beclin-1, p62 and LC3 protein expression levels following GD treatment was abrogated by inhibitor or siRNA. These observations suggested that increased Cx43 expression levels may be mediated by the ERK-autophagy signaling pathway during GD.

Previous studies have demonstrated that hyperglycemia causes downregulation of Cx43 expression levels in endothelial cells and cardiomyocytes (7,8). Hypoglycemic episodes are frequent in diabetic patients undergoing glucose-lowering therapy. However, there is limited information regarding the effect of hypoglycemia on Cx43 expression levels in H9c2 cells. The present study demonstrated that Cx43 expression levels were significantly increased following GD treatment for 2 h in H9c2 cells. The exchange rates of injury insult via gap junction channels may increase during GD, which may increase cell damage and death. In the current study, Bax protein expression levels were increased and Bcl-2 protein expression levels were decreased in the NG group compared with the control group, indicating that increased Cx43 may increase GJIC activity. Furthermore, cell viability was significantly decreased and LDH release was markedly increased under GD conditions, which was in parallel with the results of western blot analysis.

It is understood that the ERK/MAPK signaling pathway serves an essential role in regulating Cx43 expression levels (17-20). Yu et al (20) reported that advanced glycation end products upregulated Cx43 expression levels in rat cardiomyocytes via protein kinase C and ERK/MAPK signaling pathways. Activation of ERK/MAPK by epigallocatechin-gallate (EGCG) attenuated Cx43 downregulation by serum deprivation in H9c2 cells. Furthermore, inhibition of ERK by pretreatment with PD98059 may reverse the increase of Cx43 protein expression levels induced by EGCG in H9c2 cells (19). Inhibition of ERK by siRNA may suppress Cx43 expression levels induced by angiotensin II in smooth muscle cells (21). Wang et al (17) reported that Cx43 expression levels were suppressed in human aortic endothelial cells via activation of the ERK signaling pathway. The present study demonstrated that increased Cx43 protein expression levels following GD were blocked by ERK/MAPK inhibitors or siRNA-ERK. Thus, these data suggested that the ERK/MAPK signaling

pathway was involved in the regulation of Cx43 expression levels during GD.

Previous studies have provided evidence that Cx43 may be regulated by autophagy (9,22). Autophagy is a proteolytic pathway that provides cells with nutrients to adjust to environmental changes (23). Lichtenstein et al (24) observed that during starvation, Cx43 was enclosed by membrane structures containing the autophagy-associated proteins LC3 and p62. LC3 was involved in the formation of auto phagosomes and persisted for the lifespan of auto phagosome. p62 may serve as a cargo receptor for clearance of protein aggregates (25). In starved mice, Cx43 expression levels were significantly decreased at membranes, whereas they increased intracellularly (26). Furthermore, Hesketh et al (9) demonstrated that Cx43 was incorporated into the autophagosome during heart failure. The results of the present study demonstrated that protein expression levels of Beclin-1, p62 and LC3 were significantly increased during GD. It is unclear whether this increase results from an increase of autophagy flow, or damage to the degradation pathway. Notably, these protein expression levels were significantly decreased following pretreatment of U0126 or siRNA, suggesting that autophagic flow was decreased. Therefore, increased Cx43 protein expression levels may be a response to GD via activation of the autophagy pathway. Furthermore, the marked effect of U0126 on reduction of Cx43 expression levels in H9c2 cells suggested the involvement of compromised autophagosome formation. Further studies are required to confirm these interpretations.

Downregulation of Cx43 protein expression levels in the heart may limit the spread of injury insult by intercellular transmission. In a previous study, Kanno et al (10) demonstrated that Cx43-deficient mice developed smaller infracts compared with wild-type mice following coronary ligation. This may be due to decreased intercellular exchange of deleterious mediums induced by ischemia. This hypothesis was supported by Garcia-Dorado et al (27); they additionally reported that heptanol treatment may improve contractile function and decrease necrosis. These studies suggested that decreased Cx43 expression levels may limit injury by decreasing intercellular transfer of molecules under certain conditions. The results of the present study additionally indicated that increased Cx43 expression levels may be responsible for decreased cell viability and increased LDH release.

In conclusion, the present study provided evidence that GD induced upregulated Cx43 expression levels in H9c2 cells via the ERK/MAPK pathway.

Acknowledgements

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