Epigallocatechin-3 Gallate, a Green Tea Catechin, Attenuated the Downregulation of the Cardiac Gap Junction Induced by High Glucose in Neonatal Rat Cardiomyocytes

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Key Words

Epigallocatechin-3 gallate • Gap junction • High glucose • Cardiomyocytes

Abstract

Aims: The remodeling of cardiac gap junctions has been postulated to contribute to the arrhythmias in a diabetic heart. Epigallocatechin-3 gallate (EGCG), a green tea catechin, has recently been recognized for its protection in cardiovascular disease. This study investigated the effect of EGCG on the possible remodeling of gap junctions under high glucose in cultured neonatal rat cardiomyocytes. **Methods:** Cardiomyocytes pre-incubated with high glucose (30mM) were co-treated by EGCG. The expression of Connexin43 (Cx43), Cx40 and Cx45 were determined by Western blot and real-time RT-PCR. The function of cells coupling was evaluated by scrape loading dye transfer study. The Mitogen-activated protein kinases (MAPK) were quantified by Western blot. **Results:** The protein expression of Cx43 was reduced by high glucose (30mM, 72h). Addition of EGCG to high glucose treated cardiomyocytes attenuated the Cx43 reduction in a dose- and time-dependent manner and also recovered the reduced

function of cells coupling. The mRNA or protein level of Cx40 and Cx45 showed no significant change by high glucose (30mM, 72h) or EGCG co-treatment (40μM, 24h). Nor did the Cx43 mRNA level. EGCG (40μM) activated the time-dependent phosphorylated Erk, JNK and p38 MAPK. The p38 MAPK inhibitor SB203580 (10μM), however, attenuated the protective effect of EGCG. **Conclusion:** EGCG could attenuate the downregulation of gap junction induced by high glucose in cultured neonatal rat cardiomyocytes. The p38 MAPK pathway was partly involved in this effect of EGCG.

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Introduction

Cardiac gap junctions contribute to electrical coupling between cardiomyocytes and orderly electrical conduction in the heart [1]. Several studies suggested that altered gap junction expression or function could lead to the conduction disturbance and the high incidence of arrhythmias [2-3]. Cx43 is the major connexin in ventricular cardiomyocytes, whereas Cx40 and Cx45 is relatively co-expressed in atrial myocytes and the conduction system [4]. The quantity alteration or redistribution of Cx43 has been observed in diseased

myocardium such as myocardial infarction [5] or diabetic cardiomyopathy [6-9], which provides with an arrhythmogenic substrate for various arrhythmias.

It has been reported that the cardiac Cx43 expression could be up or down-regulated in streptozotocin (STZ)-induced diabetic rat model [6, 8-9], indicating the expression level of Cx43 is highly related to the diabetic cardiomyopathy. But there is no direct evidence of the remodeling of gap junctions in cultured cardiomyocytes under high glucose treatment.

Epigallocatechin-3 gallate (EGCG), a major component of green tea extracts, has recently been found to have protective role in cardiovascular disease. Several groups showed the beneficial effects of EGCG on cardiac myocytes against ischemia/reperfusion-induced apoptosis [10] and oxidative stress from cardiac hypertrophy [11]. It was reported that EGCG could protect pancreatic beta cells in diabetic rats [12]. Previous studies have also shown the potent anti-tumor activity of EGCG [13] and the pretreatment with EGCG ameliorated the Cx43-inhibitory effect of dimethylnitrosamine in Mardin-Darby canine kidney (MDCK) cells [14]. To date, however, little is known about the effect of EGCG on the protective role of cardiac gap junctions and the underlying mechanisms.

Therefore, in the present study, we aimed to investigate the expression and the function of gap junction in cultured neonatal rat cardiomyocytes under high glucose incubation, and to study the effects and the possible signal transduction pathway of EGCG co-treatment on the cardiac gap junction.

Materials and Methods

Materials

EGCG and monoclonal rabbit anti-Cx43 antibody were obtained from Sigma (St. Louis, USA). EGCG was dissolved in sterilized phosphate buffer solution (PBS, PH7.4) and used for the treatment of cells. Polyclonal rabbit anti-Cx40 antibody was purchased from Millipore (Billerica, MA, USA). Polyclonal rabbit anti-Cx45 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal rabbit antibodies including anti-Erk, anti-phospho-Erk, anti-JNK, anti-phospho-JNK, anti-p38 and anti-phospho-p38 were purchased from Cell Signaling Technology (Massachusetts, USA). The inhibitors including PD98059, SP600125 and SB203580 were obtained from Merck (Darmstadt, Germany). HRP-marked anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Kangchen (Shanghai, China). Lucifer yellow dye and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma.

Culture of neonatal rat cardiomyocytes

Cardiomyocytes were obtained from 1-2 day old Sprague-Dawley rats (provided by the experimental animal center of Zhejiang University). Heart tissues were cut into small pieces of about 1 mm³, and digested with a Ca²⁺ and Mg²⁺-free PBS containing 0.1% trypsin and 0.1% type II collagenase for 10 min at 37°C. The cell supernatant was collected and added with DMEM cell culture medium (glucose concentration: 5.5mM) containing 10% fetal bovine serum (FBS) to stop the digestive effect of trypsin and collagenase. The above steps were repeated until the tissues were completely digested. The cell suspension was then centrifugated and suspended in DMEM cell culture medium with 10% FBS in a humidified 5%CO₂/95% air atmosphere at 37°C. The fibroblast of cell suspension was reduced by pre-plating for 1 h according to differential cell adhesion [15]. 5'-BrdU (0.01mM) was also added to inhibit the growth of fibroblast during the first three days. Cell culture medium was refreshed every 48 h. All animal procedures were performed according to the *Guide for the Care and Use of Laboratory Animals* (NIH, revised 1996), and approval was granted by the university ethics review board.

Cell viability assay

A MTT-based assay was used to determine whether EGCG could affect the cell viability. Cardiomyocytes were seeded at 2x10⁴ cells per well in 96-well culture plates and incubated for 48 h. The cells were treated with either various concentration of EGCG for 24 h or various incubation period of EGCG at 40μM. The MTT solution (final concentration, 0.5mg/ml) was added to the cells for 4 h at 37°C. Then the supernatants were removed, followed by 200 μl DMSO added to each well. The absorbance was measured at a wavelength of 490nm.

Western blot analysis

Cells in 6-well plate were washed with PBS for three times, and then were incubated on ice in 100 μl lysis buffer containing 50mM Tris (PH 7.4), 150mM NaCl, 1% NP-40, 5% deoxycholic acid, 0.1% SDS, 1mM EDTA, 10mM NaF, 1mM Na₃VO₄, 1mM dithiothreitol, 1mM PMSF, 2μg/ml leupeptin for 30 min. Proteins were collected and quantified with the Bio-Rad DC Protein Assay Kit II (Bio-Rad, Hercules, USA). Samples containing equal amounts of protein were run on a 10% tris-glycine gradient gel, transferred to PVDF membranes and blocked with 5% nonfat milk in Tris-buffered solution (TBS) for 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C. After being washed three times with TBST (TBS containing 0.1% Tween 20) for 10 min each, the membranes were incubated with secondary antibodies for 1 h at room temperature. Finally the membranes were reacted with enhanced chemiluminescence (ECL) reagents and exposed by Image Quant LAS-4000 (Fujifilm, Tokyo, Japan). Band densities were determined by an image Multi-Gauge Software (Fujifilm, Tokyo, Japan).

Real-time reverse transcription-PCR (real-time RT-PCR)

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, USA), treated with RNase-free DNase

Genes	Primers	protocol
Cx43	Forward: 5'- TCTGCCTTTCGCTGTAACACT -3'	94°C 30s, 58°C 30s, 72°C 1min, 40cycle
	Reverse: 5'- GGGCACAGACACGAATATGAT -3'	
Cx40	Forward: 5'- GGAAAGAGGTGAACGGGAAG -3'	
	Reverse: 5'- GGGCCTCGAGACATAACAGTT -3'	
Cx45	Forward: 5'- GTGATGTACCTGGGATATGC -3'	
	Reverse: 5'- CCTCTTCATGGTCTCTTCC -3'	
GAPDH	Forward: 5'-ACAGTCAGCCGCATCTTCTT-3'	
	Reverse:5'-TGGAAGATGGTGATGGGATT-3'	

Table1. Oligonucleotide sequences for forward and reverse primers.

Fig. 1. Effects of EGCG on the cell viability determined by MTT assay. A. Cells were treated with EGCG at the concentration of 0, 5, 10, 20, 40 and 80 μ M for 24 h. * P <0.05 Vs. untreated cells. B. Cells were treated with EGCG at 40 μ M for 0, 6, 12, 24, 48 and 72 h, respectively. * P <0.05 Vs. control cells 72h after EGCG treatment. n=5 wells in each individual experiment; Data are mean $\pm$ SEM.

(Promega, Mannheim, Germany), and converted into cDNA using murine leukemia virus reverse transcriptase (Promega, Mannheim, Germany). The corresponding primers and the cycling conditions for PCR are presented in Table.1. The real-time PCR was performed on ABI 7500 cycler (Applied Biosystems, CA, USA) by using SYBR Premix Ex Taq reagent (Takara, Shiga, Japan). All reactions were run in triplicate with a no-template control for each run. A no-RT control was included for each sample to confirm the elimination of genomic DNA contamination. The PCR products added with loading buffer were separated on a 1.5% agarose by electrophoresis and were proved of their unity and specificity. The relative amount of target gene expression was normalized by GAPDH as an internal control and calculated by the $2^{-\Delta\Delta C_t}$ method as previously described [16].

Immunofluorescence

Cells plated on coverslips in 24-well dish were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 min. After washing with PBS for three times, cells were incubated in a blocking buffer containing 10% goat serum, followed by incubation with anti-connexin43 antibody (1:2000) in PBS containing 1% BSA overnight at 4°C. After another washing step with PBS, secondary Alexa fluor 568-labelled antibody (1:500) was applied at 37°C for 30 min. The α -actin staining was performed using primary anti- α -actin antibody (Sigma, 1:500) and subsequent secondary Alexa fluor 488-labelled antibody (1:500). After washing with PBS, the coverslips were transferred onto glass slides. Images were taken by wide-field fluorescent microscopy (Zeiss).

Dye transfer assay

Lucifer Yellow (LY), a membrane impermeable fluorescent, which could transmit from one cell to another but not diffuse through intact membrane, was used to assess the function of gap junctional intercellular communication (GJIC). Cells cultured in 35 mm dishes were incubated in 1% LY dye solution after they were rinsed with PBS. A surgical blade was used to make scrape lines through the cell monolayer. After 3 min the cells were rinsed three times with PBS to remove the remnant dye solution. The cells were then fixed by 4% paraformaldehyde for 1 min and photographed with a digital camera attached to an Olympus fluorescent microscope (magnification: 100 $\times$). The distance of the dye transferred from the scraped margin was expressed as an index of the extent of GJIC. And it was quantified by using an NIH image system.

Statistical analysis

Results were expressed as mean $\pm$ SEM. for at least three individual experiments. Statistical analysis between groups was performed by one-way ANOVA. The level of P <0.05 was considered statistically significant.

Results

Effects of EGCG on the cell viability

To determine whether EGCG could affect the viability of cardiomyocytes, cells were treated with 0, 5, 10, 20, 40, and 80 μ M EGCG for 24 h or 0, 6, 12, 24, 48

Fig. 2. Effects of EGCG on Cx43 expression in cardiomyocytes. Dose- (A) and time-dependent (B) effects of EGCG co-treatment on the Cx43 protein expression under high glucose incubation (30mM, 72h) assessed by Western blot (n=4 in each experiment); C. EGCG (40μM, 24h) attenuated the Cx43 protein downregulation induced by high glucose (Glu) (30mM, 72h) determined with immunofluorescence (n=3). Cells were stained green for α-actin (c1, c4, c7) to identify the cardiomyocytes and stained red for Cx43 (c2, c5, c8). Merged images are shown in c3, c6 and c9. D. The effects of high glucose (30mM, 72h) and EGCG co-treatment (40μM, 24h) on the Cx43 mRNA level assessed by real-time RT-PCR (n=4). E. The effects of EGCG treatment alone (40μM, 24h) on the protein and mRNA level of Cx43 determined by Western blot and real-time RT-PCR, respectively (n=3). * $P < 0.05$ Vs. Control; # $P < 0.05$ Vs. High glucose; ** $P < 0.05$ Vs. EGCG (6h, 12h, 48h); Data are mean $\pm$ SEM.

and 72 h of EGCG at a concentration of 40μM. Their viability was measured by MTT assay. As shown in Fig 1A, B, no significant cytotoxic effect was observed by the different conditions of EGCG treatment.

Effects of EGCG on the gap junction expression under high glucose incubation

Cells were co-treated by EGCG at various concentrations (10, 20, 40 and 80μM) for 24 h after high

glucose pre-incubation at 30 mM for 48 h. We found that Cx43 protein expression was reduced in cardiomyocytes subject to high glucose and this reduction was attenuated by co-treatment with EGCG in a dose-dependent manner (Fig. 2A). Cells were also co-treated by EGCG for various time courses (6, 12, 24 and 48 h) at 40μM and 30mM high glucose incubation for 72h. We noticed that EGCG could restore the Cx43 protein reduction in a time-dependent manner (peaked at 24 h) (Fig 2B). The

Fig. 3. Effects of high glucose (30mM, 72h) and EGCG (40μM, 24h) co-treatment on Cx40 (A, B) and Cx45 (C, D) expression at protein (A, C) and mRNA (B, D) level assessed by Western blot and real-time RT-PCR respectively. * $P < 0.05$ Vs. Control; $n = 4$ in each experiment; Data are mean $\pm$ SEM.

Fig. 4. Effects of EGCG on the function of GJIC between cardiomyocytes as assessed by dye transfer study. Cells were left untreated (A) or incubated with high glucose at 30mM for 72 h (B) or co-treated by EGCG at 40μM for 24 h (C). D. Quantitative analysis of dye transfer distance in different groups. * $P < 0.05$ Vs. Control; $n = 4$ in each experiment; Data are mean $\pm$ SEM.

protective effect of EGCG on Cx43 protein expression inhibited by high glucose could also be verified by immunofluorescence (Fig 2C). However, the Cx43 mRNA expression was not affected by either high glucose or co-treatment with EGCG (Fig 2D). Interestingly, EGCG alone failed to change the protein or mRNA level of Cx43 in cardiomyocytes (Fig 2E).

In addition to Cx43, Cx40 and Cx45 are also involved in the conduction of cardiomyocytes. To investigate whether the Cx40 and Cx45 expression could be affected by high glucose or EGCG, we performed the same

experiment and found there was no significant change in Cx40 and Cx45 expression at either protein or mRNA level (Fig 3A-D).

Dye transfer study of EGCG on the function of GJIC

In the control group, the cardiomyocytes remained well coupled for the presence of LY in the cardiomyocytes located at several cells' distance from the scrape margin (Fig 4A). The high glucose treatment (30mM, 72 h) significantly reduced the distance transferred by LY (Fig

Fig. 5. EGCG-induced activation of MAPKs. A. Western blot analysis of MAPKs in cultures with EGCG treatment (n=4 in each experiment). A1-A3. Showed the corresponding quantitative analysis of the phosphorylation of Erk (p-Erk), phosphorylation of JNK (p-JNK) and phosphorylation of P38 (p-P38) MAPK normalized by total MAPK and GAPDH. * $P < 0.05$ Vs. Control; # $P < 0.05$ Vs. EGCG (7.5min, 30min, 60min); B. Western blot analysis of MAPKs in cultures with EGCG (40 μM) for 15min and/or high glucose pre-incubation for 48h (n=3). B1-B3. Showed the corresponding quantitative analysis of p-Erk, p-JNK and p-P38 MAPK normalized by total MAPK and GAPDH. * $P < 0.05$ Vs. Control; Data are mean $\pm$ SEM.

4B). When the cells were co-treated by 40 μM EGCG for 24 h, the reduced distance by high glucose was recovered (Fig 4C). The corresponding data of dye transfer distance was shown in Fig 4D.

EGCG activated MAPK pathways in cardiomyocytes

Signal transduction in cardiomyocytes could be mediated by several important pathways. MAPKs act as a major signaling system in response to extracellular signals. To go insight into the changes of EGCG-induced signaling pathways, we investigated the phosphorylation level of MAPK family proteins (Erk, p38, JNK) in cardiomyocytes treated by EGCG. Cells were treated by EGCG at 40 μM for 7.5, 15, 30 and 60 min. The Western-blot analysis showed that the phosphorylation levels of Erk, JNK and p38 MAPK were significantly increased by EGCG treatment, peaked at 15 min, and then declined (Fig 5A and A1-A3).

Cardiomyocytes were then treated with EGCG (40 μM) for 15 min and/or high glucose (30mM) pre-

incubation for 48h. The phosphorylation of MAPKs could still be increased with high glucose and EGCG co-treatment, but not be activated by 48h-incubation of high glucose alone (Fig 5B and B1-B3).

The p38 MAPK pathway was involved in the protective effect of EGCG

The pre-treatment of p38 MAPK inhibitor SB203580 (10 μM) for 30min completely blocked the EGCG (40 μM, 15min) activation of phosphorylated p38 (Fig 6A). The Erk MAPK inhibitor PD98059 (20 μM) (Fig 6B) and the JNK MAPK inhibitor SP600125 (20 μM) (Fig 6C) also blocked the activation of the corresponding MAPKs. But the MAPK inhibitors alone did not affect the Cx43 expression and the dye transfer distance, as shown in Fig 6D and 6E. Cardiomyocytes pre-incubated by high glucose at 30mM for 48 h were then co-treated with EGCG (40 μM), or EGCG plus SB203580 (10 μM), or EGCG plus PD98059 (20 μM), or EGCG plus SP600125 (20 μM) for 24 h. The Western blot and the dye transfer analysis showed that the protective effect of EGCG on

Fig. 6. MAPKs inhibition and the MAPK inhibitors-induced changes of Cx43 expression and GJIC function. A-C. Western blot of the MAPKs expression in cardiomyocytes with MAPK inhibitors pretreatment for 30 min and/or EGCG treatment for 15min (n=3 in each experiment). D-E. Western blot analysis of the Cx43 expression and dye transfer distance in 24h-culture with MAPK inhibitors (n=3). F-G. Western blot analysis of the Cx43 expression and dye transfer distance in high glucose treated cardiomyocytes with EGCG co-treatment and the presence of MAPK inhibitors (n=4). * $P < 0.05$ Vs. Control (Con); # $P < 0.05$ Vs. high glucose plus EGCG co-treatment; Data are mean $\pm$ SEM.

the Cx43 expression and GJIC function inhibited by high glucose was nearly abolished by the co-treatment with SB203580, but not by PD98059 or SP600125 (Fig 6F, G). The results indicated that p38 MAPK signal transduction pathway might be partly involved in the protective effect of EGCG on Cx43 protein expression reduced by high glucose.

Discussion

In the present study, we found that the expression of Cx43 in cultured neonatal rat cardiomyocytes was reduced by high glucose. Addition of EGCG to high glucose treated cardiomyocytes attenuated the Cx43 reduction in a dose- and time-dependent manner and also recovered the reduced function of GJIC. The p38 MAPK pathway might be partly involved in the process. Interestingly, the mRNA level of Cx43 was not altered by high glucose or EGCG co-treatment. Nor did the

protein or mRNA level of Cx40 or Cx45. To our knowledge, this is the first demonstration of EGCG protecting Cx43 reduced by high glucose in cultured cardiomyocytes.

Some recent studies have focused on the expression of Cx43 in cardiac myocytes of STZ-induced diabetic rat. Compared to the control group, the amount of cardiac Cx43 was reduced in a 3-week STZ-induced diabetic rat model [6, 9] and increased in a 12-week diabetic rat model [8]. Nygren et al [7] found that the immunoblotting of Cx43 showed no change in the diabetic rat heart after 7-14 days of STZ injection, and the enhanced Cx43 lateralization was recognized, which may be associated with non-functional gap junctions. These discordant results may be due to the different STZ concentrations, or the different time courses after STZ injection, or the effect of advanced glycation end products (AGE) and hypertriglyceridemia [17]. For the miscellaneous factors existed in the animal model, the neonatal rat cardiomyocytes were cultured in our present study to

examine the expression of gap junctions under high glucose at 30mM, which is a hyperglycemia model frequently used in cultured cells[18-19]. Here we observed that high glucose impaired the expression of Cx43 protein as well as the function of GJIC in cardiomyocytes. It has been described that the cardiac Cx43 down-regulation could produce shorter action potential duration (APD) [20] and lead to significant slowing of action potential (AP) propagation and delayed calcium transient between cells [21], which would contribute to lethal arrhythmias or desynchronization of a failing heart. It might account for the high susceptibility to ventricular fibrillation and myocardial contractile dysfunction in a diabetic heart [22].

EGCG has been recently recognized for its protection against cardiovascular disease. Besides its anti-oxidant effect of cardiac hypertrophy [11, 23-24], EGCG has been reported to reduce the myocardial infarction and attenuate the myocyte apoptosis in the isolated rat heart subject to ischemia/reperfusion injury by inhibition of STAT-1 activation [10] or by mitochondrial K(ATP) channel activation [25]. It could also enhance intracellular Ca^{2+} concentration in rat cardiomyocytes via activation of Na^+/H^+ and $\text{Na}^+/\text{Ca}^{2+}$ exchangers [26] or in aortic smooth muscle cells via specific Ca^{2+} channels [27]. In our present study, we examined the effect of EGCG on cardiac gap junction inhibited by high glucose. Although Cx43 mRNA level was not altered by EGCG, the protein expression of Cx43 and the function of GJIC was significantly recovered by EGCG co-treatment. It has been reported that the decrease of Cx43 protein expression in myocardial ischemia or hypoxia could be alleviated by antiarrhythmic peptide AAP10 [28] or acetylcholine [29]. But no direct evidence mentioned any agent in protecting the remodeling of Cx43 in a diabetic heart. Our result indicated the beneficial effect of EGCG as a protective agent against cardiac Cx43 reduction by high glucose, which might provide with some clues in preventing lethal arrhythmias in a diabetic heart.

Interestingly, the mRNA level of Cx43 in our study was not altered by either high glucose or EGCG, which suggested a post-transcriptional modification in cardiomyocytes. A previous study also demonstrated the similar quantification of Cx43 mRNA between diabetic and control rat heart [8]. Recently, Lin et al found that reduced Cx43 protein in diabetic heart could be alleviated by either lysosomal (NH_4Cl , Leupeptin) or proteasomal inhibitor (ALLN) [6], indicating the possible process of the acceleration of the Cx43 proteolytic degradation. It was also reported that high glucose down-regulated the

GJIC in retinal endothelial cells by enhancing Cx43 degradation via a proteasome-dependent mechanism [30]. Therefore, we proposed that high glucose might downregulate Cx43 by accelerating the Cx43 proteolytic degradation in cardiomyocytes and EGCG might reverse this effect by attenuating the Cx43 protein degradation, but not by the stimulation of Cx43 mRNA level.

MAPKs are a family of proteins that act as a major signaling system in cells to response to extracellular signals. Previous studies have demonstrated that MAPKs were involved in regulating cell surface molecules [31] and partly activated by EGCG treatment in endothelial cells [32]. In our study, EGCG increased the time-dependent phosphorylation of MAPK family proteins (Erk, p38, JNK) in cardiomyocytes. And the effect still existed with high glucose pre-incubation for 48h. As the upstream signals of MAPK pathways, their transient phosphorylation could activate the downstream signals leading to cascade effect and prolonged existence of the ultimate gene products, which participated in the regulation of protein expression [33-34]. Interestingly, MAPKs kept inactivated with high glucose incubation alone for 48h. But it could not exclude the possibility that the transient MAPKs activation might exist shortly after high glucose treatment or some other signal pathways participated in the possible Cx43 protein degradation by high glucose, which still needed further investigation.

In our study, the MAPK inhibitors (SB203580, PD98059 and SP600125) effectively abolished the EGCG-induced MAPKs activation. The treatment of MAPK inhibitors alone did not alter the Cx43 expression and the function of GJIC, which was consistent with previous studies demonstrating that neither PD98059 nor SB203580 had any effect on Cx43 abundance or phosphorylation in cardiomyocytes [35-36]. Recently Matsuyama et al found that the knock down of Cx43 by siRNA in cardiomyocytes was accompanied with a significant decrease of p-p38 MAPK [37]. But we found that the Cx43 protein downregulation by high glucose was not accompanied with altered p-p38 MAPK. The discordant result might be due to the post-transcriptional modification of Cx43 expression by high glucose in our study. In addition, the protective effect of EGCG on the reduced cardiac Cx43 by high glucose was nearly abolished by the pretreatment of p38 MAPK inhibitor SB203580, but not by PD98059 or SP600125. As EGCG did not alter the Cx43 mRNA level either, it suggested that the p38 MAPK activation by EGCG in cardiomyocytes might not directly regulate the Cx43 expression via the stimulation of Cx43 mRNA level, but

was partly involved in EGCG-induced protein stabilization effect of attenuating the Cx43 degradation by high glucose.

Cx40 and Cx45 are the other two mainly expressed connexins in cardiomyocytes and exist in limited regions as atrial muscle and conduction system in an adult heart [4]. We did not find any significant change in Cx40 or Cx45 at either mRNA or protein level exposed to high glucose or EGCG, which differs from another study demonstrating that they were both over-expressed at early stages of diabetic cardiomyopathy [9]. It could be explained for the different spatial distribution and miscellaneous factors in vivo models. In another vitro study [38], only Cx43, but not Cx40 or Cx45 was increased by TNF- α stimulation in cultured neonatal rat cardiomyocytes, since Cx40 and Cx45 showed very low expression at the detection limit, which may also partly account for our result. The present work further testified that the reduced function of GJIC by high glucose was due to the reduction of Cx43, but not Cx40 or Cx45. It confirms the predominant function of Cx43 in cardiac GJIC.

In summary, the present study demonstrated that EGCG attenuated the reduction of Cx43 protein and GJIC in cardiomyocytes under high glucose partly through p38 MAPK signal transduction pathway. The mRNA level of Cx43, Cx40 and Cx45, however, remained constant with high glucose or EGCG. It suggested a therapeutic strategy to protect the cardiac gap junctions against remodeling by hyperglycemia. Limitations existed in the lack of mechanism investigation of Cx43 protein downregulation by high glucose and the lack of cell viability assessment in dye transfer assay. Further studies are needed to testify the effect of EGCG in vivo models and explore the effect of miscellaneous factors like AGE on the gap junction remodeling in a diabetic heart.

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