The effect of small G protein signaling modulator 3 for hypoxic cardiomyocytes

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The last, I appreciate to my parents, my wife and lovely son who are always encouraging me.

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ABSTRACT

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Connexin 43 (Cx43), a ubiquitous connexin expressed in the heart and skin, is associated with a variety of hereditary conditions. Therefore, the characterization of Cx43-interacting proteins and their dynamics is important to understand not only the molecular mechanisms underlying pathological malfunction of gap junction-mediated intercellular communication but also to identify novel and unanticipated biological functions of Cx43. In the present

study, we observed potential targets of Cx43 to determine new molecular functions in cardio-protection. MALDI-TOF mass spectrometry analysis of Cx43 co-immunoprecipitated proteins showed that Cx43 interacts with several proteins related to metabolism. In GeneMANIA network analysis, SGSM3, which has not been previously associated with Cx43, was highly correlated with Cx43 in heart functions, and high levels of SGSM3 appeared to induce the turnover of Cx43 through lysosomal degradation in myocardial infarcted rat hearts. Moreover, we confirmed that lysosomal degradation of Cx43 is dependent upon the interaction between SGSM3 and Cx43 in H9c2 cardiomyocytes. Additionally, we investigated the protective effects of kenpaullone on cardiomyocytes following H_2O_2-induced oxidative stress mediated by the interaction of SGSM3 with Cx43. We found that the gap junction protein Cx43 was significantly down-regulated in an H_2O_2 concentration-dependent manner, whereas expression of SGSM3 was up-regulated upon H_2O_2 exposure in H9c2 cells. The effect of kenpaullone pretreatment on H_2O_2-induced cytotoxicity was evaluated in H9c2 cells. H_2O_2 markedly increased the release of lactate dehydrogenase (LDH), while kenpaullone pretreatment suppressed LDH release in H9c2 cells. The functional importance of the interaction between SGSM3 and Cx43 was confirmed by

results showing that Cx43 expression was enhanced by SGSM3 siRNA knockdown in H9c2 cells. Moreover, kenpaullone pretreatment significantly reduced ROS fluorescence intensity and significantly down-regulated the level of apoptosis-activating genes (cleaved caspase-3, cleaved caspase-9 and cytochrome C), autophagy markers (LC3A/B), and the Cx43-interacting partner SGSM3. In summary, the results of this study elucidate the molecular mechanisms in which Cx43 with SGSM3 is degraded in myocardial infarcted rat hearts, which may contribute to the establishment of new therapeutic targets to modulate cardiac function in physiological and pathological conditions. In addition, kenpaullone plays a role in protecting cardiomyocytes from oxidative stress and that the turnover of Cx43 through SGSM3-induced lysosomal degradation underlies the anti-apoptotic effect of kenpaullone.

Key words: cardiomyocytes, connexin 43, gap junction, myocardial infarction, oxidative stress, SGSM3

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I. INTRODUCTION

Gap junctions (GJs) are aggregates of intercellular membrane channels, and GJ communication promotes apoptosis in a connexin-type-dependent manner.[1, 2] Connexin (Cx) 43, the major gap junction-forming protein in the adult cardiac ventricles, plays a pivotal role in mediating tissue injury, yet few studies have addressed the role of cardiac Cx43 in modulating myocyte death.[3] Recent

literature has suggested that Cx43 is present in the mitochondria and may play a role in mediating the cardioprotective effect of ischemic preconditioning.[4-6] Emerging evidence has shown that regulation of Cx43 turnover and degradation by the proteasomal or lysosomal pathway mediates cell death in various heart diseases.[6-8]

In addition to its role in GJIC, Cx43 has shown channel-independent functions. Indeed, many reports have suggested that Cx43 regulates other cellular mechanisms, including the cell cycle, differentiation and proliferation.[9-11] Cx43 in cardiomyocyte mitochondria is localized at the mitochondrial inner membranes, where it has been implicated in enhanced ischemic preconditioning response. Hence, researchers found that during myocardial ischemia, the mitochondrial Cx43 increases, which could contribute to maintaining the closed state of the mitochondrial permeability transition pore, delaying the release of apoptotic proteins such as cytochrome c and thus reducing ischemia/reperfusion (I/R) injury.[5, 12]

Increased degradation of Cx43 is one contributing factor to the GJIC impairment underlying heart disorders and could play an important role in GJ remodeling in response to cardiac injury.[6] The function of Cx43-containing channels depends on Cx43-interacting proteins, which through the interaction

itself, or by mediating post-translational modifications, can modulate the subcellular distribution and activity of Cx43.[13, 14] Consequently, we aimed to understand how interacting partners regulate GJIC in physiological and pathological conditions and the role played by non-canonical functions of Cx43.[15]

Oxidative stress induced by hydrogen peroxide (H_2O_2) causes several human diseases, including inflammation, heart disease, and cerebrovascular disease, through multiple mechanisms.[16] Moreover, reactive oxygen species (ROS) and free radicals play central roles in cardiac pathophysiology when the heart is subjected to significant oxidative stress following ischemia due to heart surgery and heart diseases.[17, 18] Thus, hydrogen peroxide has been widely used as an external agent to induce oxidative stress in models of ischemic cardiac conditions.[19-21] Indeed, we applied different dosages of H_2O_2 to determine whether Cx43 degradation in response to higher expression levels of SGSM3 is mediated by lysosomal degradation under oxidative stress.

Kenpaullone acts on a biologically meaningful and pharmacologically "druggable" target for the inhibition of glycogen synthase kinase 3β (GSK-3β) and several cyclin-dependent kinases (CDKs).[22] Recently, kenpaullone has been reported to exert cardioprotective effects on hypoxia-induced cardiomyocyte

death *in vitro*.[23] However, the underlying protective effects of kenpaullone in cardiac ischemic conditions have not been fully characterized.

Although several interacting partners of Cx43 have been identified in GJ-dependent and GJ-independent functions, to date, the partner proteins of the cardio-protective role of Cx43 are unknown. The aim of the present report was to identify new molecular partners of Cx43 that participate in myocardial infarction (MI) in rat heart, which ultimately may lead to the establishment of new therapeutic targets to modulate cardiac homeostasis/protection. Additionally, we hypothesized that the effects of kenpaullone are mediated by inhibition of expression of SGSM3, as high levels of SGSM3 appeared to induce the turnover of Cx43 through lysosomal degradation in infarcted rat hearts. Furthermore, our data reveal for the first time that kenpaullone is able to improve cardiomyocyte survival following hydrogen peroxide-induced oxidative stress through inhibition of SGSM3 against Cx43 degradation.

II. MATERIALS AND METHODS

1. MI rat model and histological analysis of fibrosis area

All experimental procedures for animal studies were approved by the

Committee for the Care and Use of Laboratory Animals of Catholic Kwandong University College of Medicine (CKU01-2015-003-1) and performed in accordance with the Committee's Guidelines and Regulations for Animal Care. Seven-week-old male Sprague-Dawley rats (220 ± 30 g) were used for in vivo experiments. After anesthetization via intraperitoneal injection of zoletil (30 mg/kg) and xylazine (10 mg/kg), rats were ventilated via the trachea using a ventilator (Harvard Apparatus, Holliston, MA, USA) and then were subjected to surgically induced MI.[24] MI was produced by surgical occlusion of the left anterior descending coronary artery by ligation using a 7-0 Prolene suture (Covidien, Dublin, Ireland). Animals were euthanized after 7 days to obtain cardiac tissue, which were fixed with 4% formaldehyde. After the paraffin embedding, the slide was made to a thickness of 5 μm. Masson's trichrome staining was performed according to the manufacturer's instructions (Cat. No. SDHT15, Sigma-Aldrich). The slides were observed by virtual microscopy (BX51/dot slide; Olympus, Tokyo, Japan).[25]

2. Cell culture

A rat cardiomyocyte line, H9c2 (American Type Culture Collection), was cultured in Dulbecco's modified Eagle's medium (DMEM) (American Type

Culture Collection) containing 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin at 37℃ under humidified conditions (5% CO_2 atmosphere).

3. Cell viability and cytotoxicity assay

H9c2 cells were seeded 24 h prior to treatment with H_2O_2 at a density of 3×10^4 cells/well in a 96-well plate. After 24 h, H_2O_2 was applied to the cells at different concentrations (50, 100, 200, 300 μM) and incubated for 24 h. Then, H9c2 cell viability was measured using Ez-Cytox (DOGEN, Seoul, Korea), and the cytotoxicity of H_2O_2 in H9c2 cells was determined by an LDH (lactate dehydrogenase) Cytotoxicity Detection Kit (Takara, Nojihigashi, Kusatsu, Shiga, Japan) following the manufacturer's instructions.

4. ROS detection assay

H9c2 cells were plated 24 h prior to H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) treatment at a density of 1×10^6 cells/well in 6-well plates, and ROS was induced after a 3-h treatment with 50, 100, 200, or 300 μM H_2O_2 with/without kenpaullone pretreatment (Sigma-Aldrich) for 1 and 2 h followed by exposure to 50 μM DCF-DA (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37℃ in

the dark. Green fluorescence was detected using a BD AccuriC6 Cytometer (BD Biosciences, Piscataway, NJ, USA).[26]

5. Co-immunoprecipitation (Co-IP)

For identification of Cx43-interacting partner proteins in normal and MI hearts, Co-IP was performed with slight modifications as described previously.[27] Input proteins containing 500 μg of protein were incubated with 10 μl of anti-Cx43 antibody (Cell Signaling) at 4℃ for 3 h on a rotary shaker, after which 20 μl of A/G PLUS agarose conjugate suspension (Santa Cruz Biotechnology) was added and allowed to mix at 4℃ for 12 h on a rotary shaker. For the negative control sample, control goat IgG (Santa Cruz Biotechnology) was used. After Co-IP, all samples were separated by 8-12% SDS-PAGE, and the samples were then transferred to a PVDF membranes for immunoblot analysis.

6. Immunoblot analysis

The immunoblot analysis was performed as described in our previous studies.[8, 26] Primary polyclonal antibodies against caspase-3 (Merck KGaA, Darmstadt, Germany), caspase-9 (Merck KGaA), Cx43 (Merck KGaA),

cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA), LC3A/B (Merck KGaA), SGSM3 (Antibodies-Online), ZO-1 (Millipore), or β-actin (Santa Cruz Biotechnology) and secondary antibodies (horseradish peroxidase-conjugated anti-goat IgG, anti-mouse IgG or anti-rabbit IgG; Santa Cruz Biotechnology) were used to detect the proteins of interest. The results were visualized using an enhanced chemiluminescence (ECL, Western Blotting Detection kit, GE Healthcare, Sweden) system, and the band intensities were quantified using ImageJ software (NIH).

7. Real-time RT-PCR

The level of each gene transcript was quantitatively determined using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Total RNA was isolated from rat hearts using TRIzol reagent (Life Technologies, Frederick, MD, USA), and reverse-transcription was performed using a Maxime RT Premix kit (iNtRON Biotechnology, Seongnam, Korea). A SYBR Green Dye system (SYBR Premix Ex Taq [Tli RNase Plus]) with a ROX reference dye (TaKaRa Bio Inc., Foster City, CA, USA) was used to perform real-time RT-PCR. The level of each gene transcript (Gja1, Tjp1, and Sgsm3) was normalized to Gapdh transcript levels, and relative changes in gene

expression were quantified using the ΔΔCT method.[8, 26] Primers were designed using Primer3 and BLAST, and the primer set sequences are listed in Table 1.

Table 1. Sequences of primers used for quantitative real-time RT-PCRs

Genes		**Primer sequence (5′ - 3′)**
Gap junction and target		
Gja1	F	CTCACGTCCCACGGAGAAAA
	R	CGCGATCCTTAACGCCTTTG
Tjp1	F	AGACAATAGCATCCTCCCACC
	R	TAGGGTCACAGTGTGGCAAG
Sgsm3	F	CTGACACAGGGCAGATGAAG
	R	TCATGTGCTGTGGACGATGG
Internal control		
Gapdh	F	TCTCTGCTCCTCCCTGTTCTA
	R	GGTAACCAGGCGTCCGATAC

[a)] F, sequence from sense strands; [b)] R, sequence from antisense strands

8. Multicolor immunofluorescence staining

To analyze the target protein expression patterns in rat hearts under pathological conditions, the tissue slides were washed with PBS and subjected to permeabilization in 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). The tissue slides were washed with PBS three times, blocked with 1% BSA in PBS-T for 1 h and then incubated with polyclonal anti-Cx43 antibody

(1:100 dilution, Cell Signaling) and anti-SGSM3 antibody (1:100 dilution, Antibodies-Online) overnight at 4℃. The slides were then washed three times with PBS and incubated with rhodamine-conjugated anti-goat IgG (1:500 dilution, EMD Millipore) against SGSM3, FITC-conjugated anti-rabbit IgG (1:500 dilution, Vector Laboratories) against Cx43. DAPI (Invitrogen) was used to stain cell nuclei. The prepared slides were observed using an LSM700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Image acquisition was performed using Zen software (Carl Zeiss).

9. Transient knockdown of siRNAs

For knockdown (KD) of *Gja1 and Sgsm3*, target-specific commercial AccuTarget siRNAs (BIONEER, Daejeon, Korea) [*Gja1* siRNA no. 1647500: sense (5′-3′), GAGAGUGUUCUUUAUCCAA (dTdT); antisense (5′-3′), UUGGAUAAAGAACACUCUC (dTdT); *Sgsm3* siRNA no. 1752125: sense (5′-3′), CUGAUACAGUCGGAGAACU (dTdT); antisense (5′-3′), AGUUCUCCGACUGU AUCAG (dTdT)] were designed, and a negative control was used. The H9c2 cells (1×10^5 cells per dish in a 35-mm dish) were transiently transfected with siRNA (50 nM per dish) and transfection agent (7.5 μl per dish) using the TransIT-X2 Dynamic Delivery System (Mirus Bio LLC,

Madison, WI, USA).

10. Statistical analysis

All experimental results were compared using independent t-test or one-way analysis of variance (ANOVA) in the Statistical Package of Social Science (SPSS, version 17) program. The data are expressed as the mean ± SEM. A protected least-significant difference (LSD) test, which is a method for analyzing multiple comparisons by single-step procedures in one-way ANOVA, was used to identify significant differences between means ($p < 0.05$).

III. RESULTS

1. Expression of *Cx43* and *ZO-1* in normal and MI rat hearts

As expected, after Masson's trichrome staining, distinct blue collagen fibers were detected in MI rat hearts (Figure 1A). For identification of differentially expressed GJ proteins between normal and MI rat heart, two GJ genes/proteins (*Gja1*/Cx43 and *Tjp1*/ZO-1) were verified by Western blot and RT-PCR analysis (Figure 1B). We observed that the two GJ proteins were significantly down-regulated in MI rat hearts. Furthermore, Cx43 GJ proteins

were abundantly expressed in the intercalated discs in normal heart, whereas expression of Cx43 was down-regulated in the MI heart group as shown by immunofluorescence staining (Figure 2).

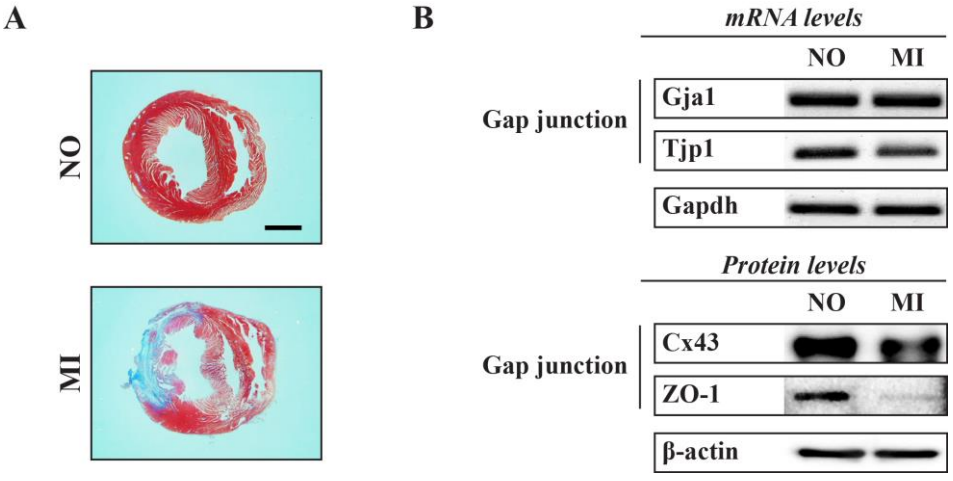

Fig. 1. Comparison of GJ proteins (Cx43, ZO-1) between normal (NO) and MI rat hearts. (A) Masson's trichrome staining, (B) quantitative real-time RT-PCR (Gja1, Tjp1) and immunoblot analysis. Significant differences between normal and MI rat hearts were determined via independent t-test, with *p* values indicated as *$p<0.05$ and **$p<0.01$.

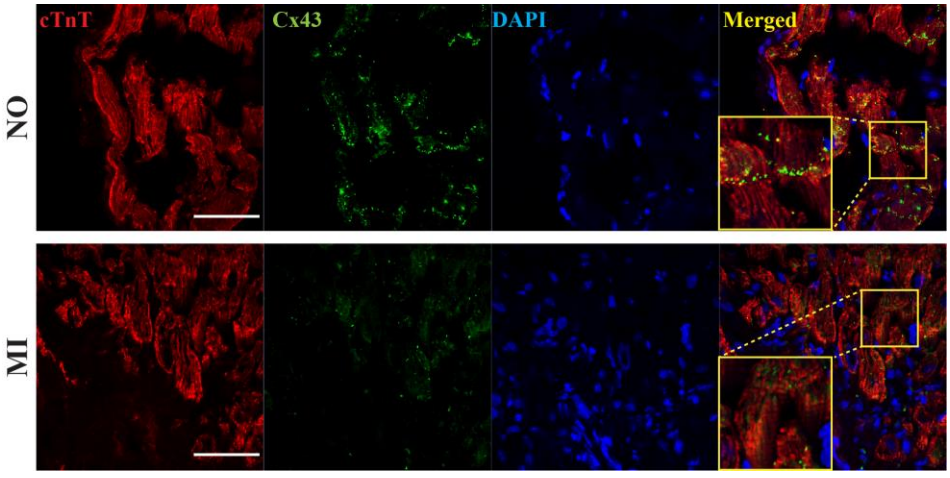

Figure 2. Cx43 immunohistochemical staining. Sections were observed at 200× magnification, and the scale bar represents 20 μm.

2. Identification of Cx43-interacting proteins

We hypothesized that new molecular effectors could increase levels of GJIC or cell survival by new molecular pathways. To investigate potential partners related to this interaction, Co-IP assays were performed with total proteins from normal and MI rat hearts (Figure 3A). Among the changed bands in silver-stained gels, seven bands were differentially regulated between the groups, and four bands were identified using peptide mass fingerprinting (PMF) (Table 2). In addition, to predict gene interactions between Cx43 and its

interacting partners, we used a predictive web interface, GeneMANIA. The GeneMANIA results suggested that SGSM3 was strongly associated with Cx43 in rat MI hearts (Figure 3B).

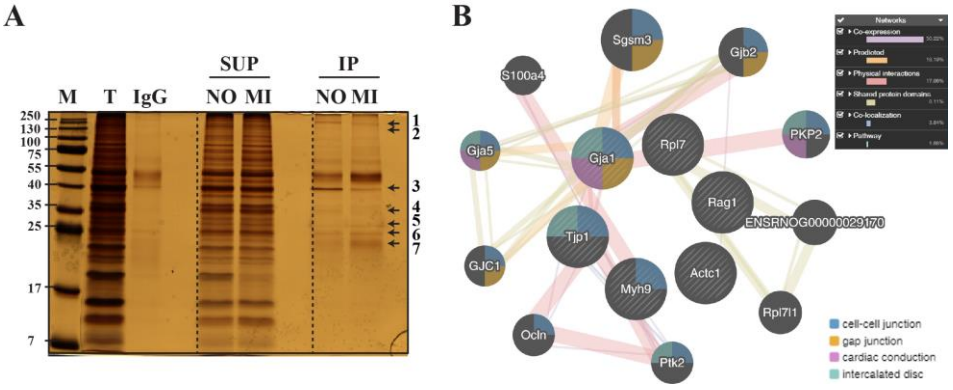

Figure 3. Discovery of Cx43 partner proteins. (A) Representative silver-stained images of co-immunoprecipitated sample with anti-Cx43 antibody from normal and MI rat hearts. Proteins were identified by PMF analysis and are indicated by arrows along with their abbreviated names. Full names of proteins are presented in the abbreviation section. (B) GeneMANIA showing the results using the following advanced parameters: molecular function-based. Orange line shows a network of predicted protein-protein interactions.

Table 2. List of identified proteins showing differential expression between normal and MI rat hearts

ID [a)]	Gene name	Description	Acc. no.	Nominal mass (*Mr*) [d)]	Calculated PI	Score [e)]
1	Myh9	Myosin-9	gi|806549497	226273	5.54	163
2	ND [f)]					
3	Actc1	Actin, alpha cardiac muscle 1 precursor	gi|4885049	41992	5.23	67
4	ND					
5	Rpl7	Ribosomal protein L7, partial	gi|206736	29256	10.7	124
6	ND					
7	Rag1	RAG1, partial	gi|319839206	46549	9.82	67

a) Protein ID indicates the numbers in Co-IP-images in Fig. 2.
b) Acc. no. is a NCBInr database accession number.
c) Acc. no. is an entry in the UniProt/SWISS-PROT database.
d) The nominal mass is the integer mass of the most abundant naturally occurring stable isotope of an element. The nominal mass of a molecule is the sum of the nominal masses of the elements in its empirical formula.
e) Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 62 are significant ($p<0.05$).
f) ND is not detected.

3. Validation of a Cx43-interacting partner (SGSM3) and apoptosis/autophagy markers in normal and MI rat hearts

To verify the expression levels of Cx43-interacting partners between normal

and MI rat heart, we evaluated two GJ genes and SGSM3 by real-time PCR analysis (Figure 4). As shown in Fig. 3A, SGSM3 gene expression was up-regulated in MI hearts compared with their expression in the control hearts. However, the two GJ genes (*Gja1*, *Tjp1*) were down-regulated in the MI group. As shown in Figure 5, Western blot analysis indicated that apoptosis and autophagy signaling markers increased in MI rat heart, possibly due to GJ turnover by the internalization of endosome, as reported by other researchers. As a result, the expression level of cleaved Caspase 3, cleaved Caspase 9, Cytochrome C, and LC3A/B showed significant increases in MI hearts. Moreover, intensity of the localization pattern was used to evaluate the relative levels of SGSM3 with Cx43 between normal and MI rat hearts by multicolor immunofluorescence staining (Figure 6). Consistent with the previous results, high expression of SGSM3 was observed in MI rat hearts, while low Cx43 expression was detected in infarcted hearts. Based on these results, we hypothesized that SGSM3 interacts with Cx43, indicating that SGSM3 plays an important role in GJIC or cell survival in rat heart. Furthermore, recent studies have detected the expression of connexins in subcellular locations other than the plasma membrane, including the cell nucleus, where it has been suggested that Cx43 influenced cell growth and differentiation.[28] Moreover, several pathways

have been implicated in connexin degradation, including two proteolytic pathways (proteasomal and lysosomal-based pathways).[29]

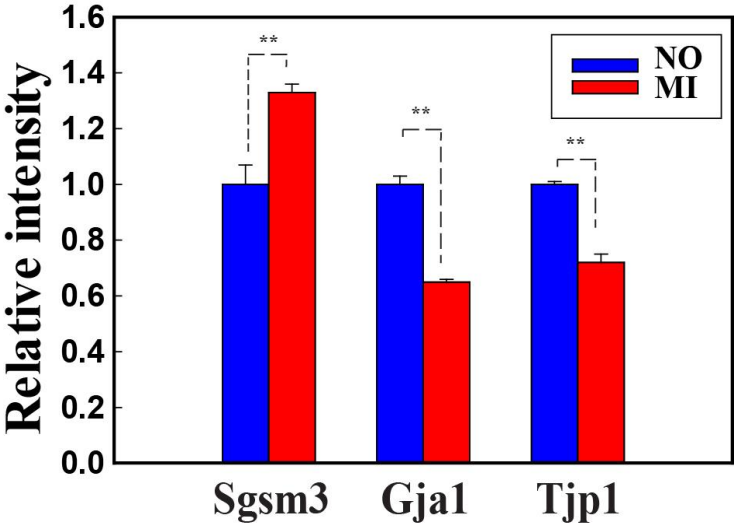

Figure 4. Validation of the protein-protein interaction of Cx43 with SGSM3 protein between normal and MI rat hearts. Quantitative real-time RT-PCR (Gja1, Tjp1, Sgsm3). Independent t-test was used to calculate statistical significance between groups, where p-value is $^{*}p<0.05$, $^{**}p<0.01$.

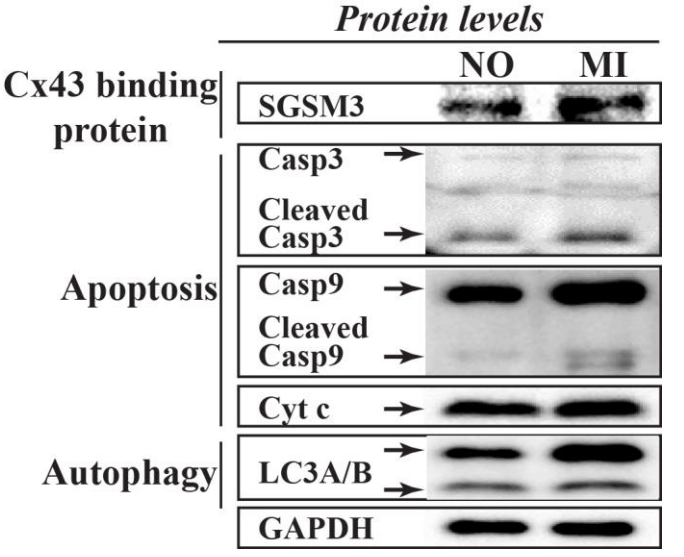

Fig. 5. Validation of the protein-protein interaction of Cx43 with SGSM3 protein between normal and MI rat hearts. Differentiation of apoptosis and autophagy markers between normal and MI rat hearts.

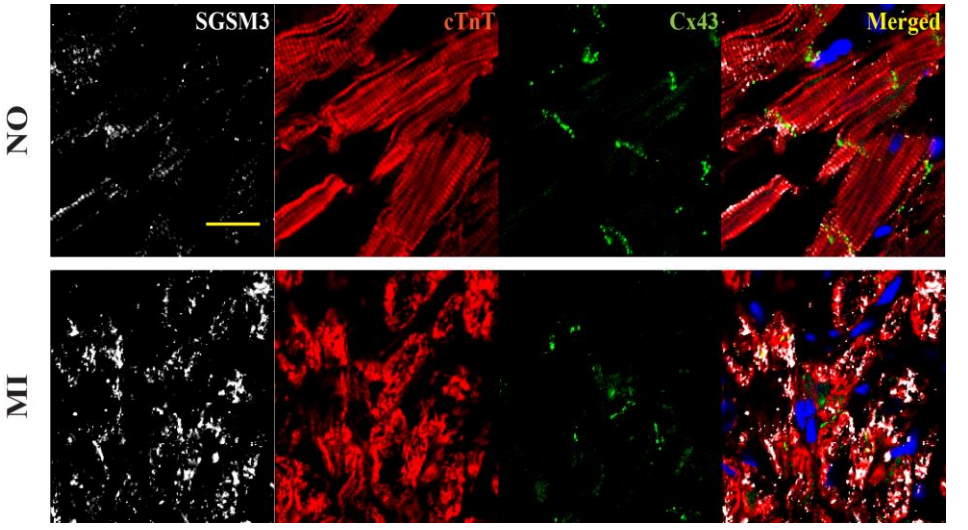

Fig. 6. Validation of the protein-protein interaction of Cx43 with SGSM3 protein between normal and MI rat hearts. Co-localization of Cx43 and SGSM3 proteins by double immunofluorescence. Multicolor labeling with Cx43 (green), SGSM3 (white), and cTnT protein (red) indicates that these markers are co-expressed in rat hearts. Nuclei were stained with DAPI (blue). Sections were observed at 400× magnification, and the scale bar represents 20 μm.

4. *Cx43*/*Gja1* turnover induced by *SGSM3* via autophagy/lysosomal pathways in cardiomyocytes

To assess the function of Gja1 and SGSM3 in rat cardiomyocytes, we performed Gja1/Sgsm3 KD experiments for 24 h in H9c2 cells, and cell viability was determine using CCK-8 assays. Gja1 KD resulted in a significant decrease in cell viability under normoxic and hypoxic conditions (Figure 7). Interestingly, KD of Sgsm3 by siRNA transfection in H9c2 cells under hypoxic conditions protected Cx43 from degradation. Additionally, protein levels of apoptosis/autophagy proteins and Cx43 were compared between the siRNA knockdown cells and the control cells (Figure 8). Western blot results showed that Cx43 levels were stabilized by SGSM3 siRNA transfection in H9c2 cells. In addition, the expression of apoptosis/autophagy proteins was significantly inhibited by SGSM3 siRNA transfection in H9c2. Taken together, our results suggest that knockdown of SGSM3 protected Cx43 from lysosomal/autophagic degradation and probably impaired the interaction of Cx43 with LC3.

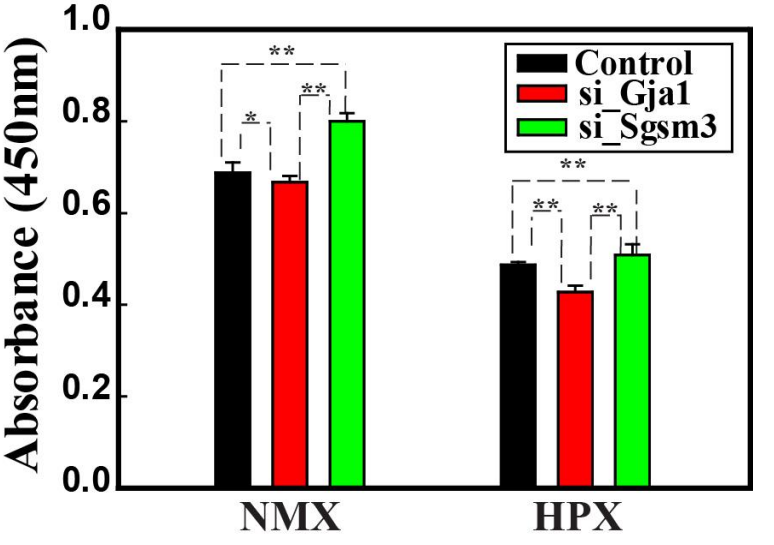

Figure 7. Effects of Cx43 and SGSM3 knockdown on survival of H9c2 between normoxic (NMX) and hypoxic (HPX) conditions. Cell viability results are representative of three independent experiments. Significant differences between the NMX and HPX groups were determined via ANOVA, with *p* values indicated as *$p<0.05$ and **$p<0.01$.

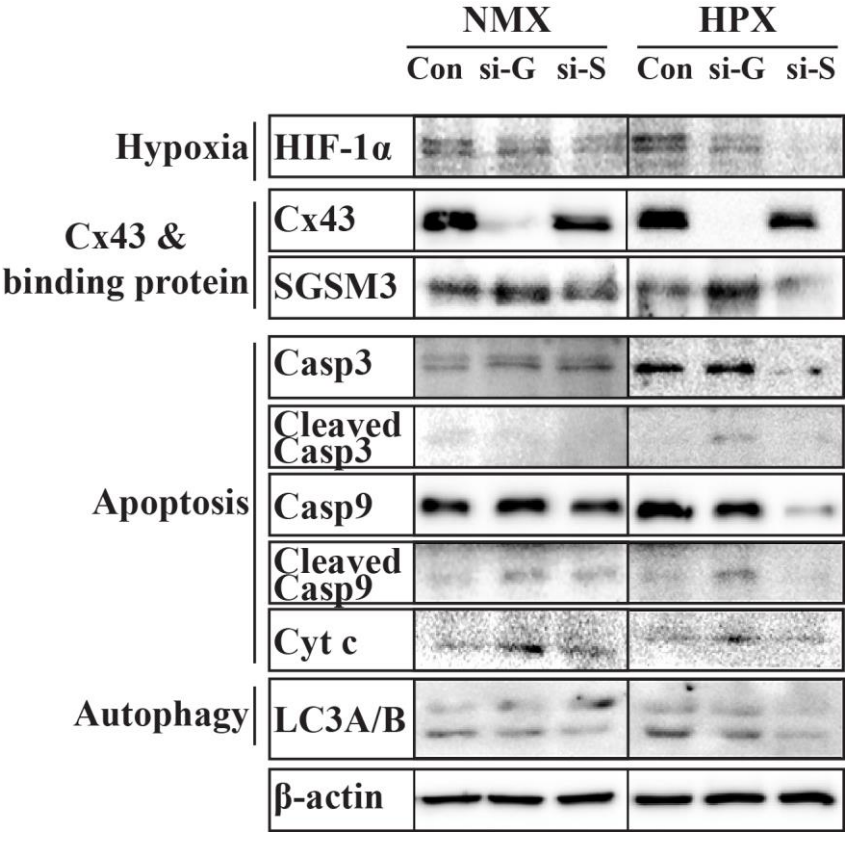

Figure 8. Effects of Cx43 and SGSM3 knockdown on the expression level of apoptosis/autophagy proteins in H9c2 between normoxic (NMX) and hypoxic (HPX) conditions. Immunoblot analysis results.

5. Effects of the H_2O_2 concentration and kenpaullone on H9c2 cells

To explore the effect of kenpaullone on H_2O_2-induced injury in H9c2 cells, we tested different doses (0-300 μM H_2O_2) at which cytotoxicity develops within 3 h following H_2O_2 exposure in H9c2 cardiomyocytes (Figure 9A). The maximum reduction was 73 ± 6% of the control group with 300 μM H_2O_2 treatment. We chose to use 300 μM H_2O_2 in our subsequent experiments based on this result. H_2O_2-induced H9c2 cells showed remarkably higher ROS fluorescence intensity than controls depending on the H_2O_2 concentration (Figure 9B). To determine whether Cx43 and SGSM3 are differentially expressed at different concentrations of H_2O_2, expression levels of the two proteins (Cx43 and SGSM3) were verified by Western blot analysis (Figure 9C). Cx43 GJ protein expression was significantly down-regulated in a H_2O_2 concentration-dependent manner, whereas SGSM3 expression was up-regulated upon H_2O_2 exposure in H9c2 cells. We next investigated cell survival between H9c2 cells with and without kenpaullone pretreatment (KP1, pretreatment for 1 h; KP2, pretreatment for 2 h) under oxidative conditions (300 μM H_2O_2) (Figure 10A and 10B). To evaluate whether kenpaullone is cytotoxic to H9c2 cells, we determined the viability of cells pretreated with kenpaullone (10 μM) for 1-2 h

using the CCK-8 kit. Cell viability was significantly reduced by H_2O_2 treatment, but kenpaullone pretreatment increased cell viability compared with H_2O_2 treatment only. To further explore the role of kenpaullone in preventing H_2O_2-induced cytotoxicity in H9c2 cells, we measured LDH release from the cytosol to culture medium. H_2O_2 markedly increased the release of LDH, while kenpaullone pretreatment suppressed LDH release in H9c2 cells (Fig. 1E). Moreover, kenpaullone pretreatment significantly reduced ROS fluorescence intensity (Figure 10C). These results revealed that kenpaullone protected H9c2 cardiomyocytes against H_2O_2-induced cytotoxicity.

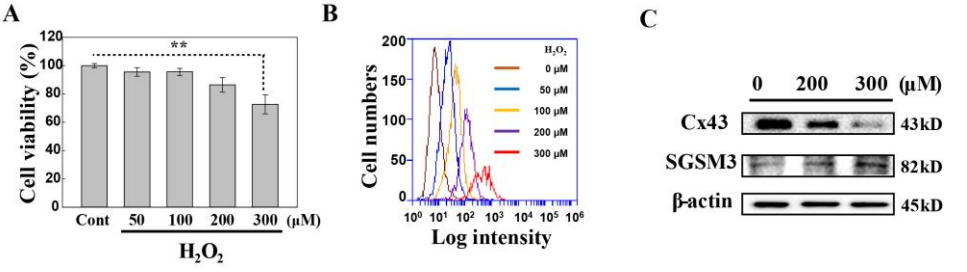

Figure 9. Effects of ROS production following H_2O_2 treatment on H9c2 cell.
(A) Cell viability and (B) ROS production following H_2O_2 treatment, and (C) representative immunoblots of Cx43 and SGSM3. Significant differences were determined by ANOVA, with *p* values indicated as *p<0.05 and **p<0.01

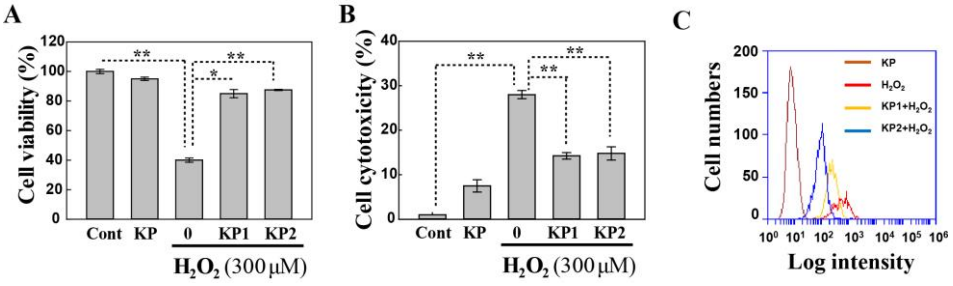

Figure 10. Effects of kenpaullone on H_2O_2-induced H9c2 cell injury. (A) protective effect of kenpaullone, (B) anti-cytotoxic effect from kenpaullone treatment, and (C) ROS production with kenpaullone treatment. Significant differences were determined by ANOVA, with *p* values indicated as *p<0.05 and **p<0.01.

6. Protective effect of kenpaullone on H_2O_2-induced H9c2 cells via changes in apoptosis/autophagy marker and Cx43-interacting partner SGSM3 expression

Subsequently, we further investigated the effect of kenpaullone pretreatment on H_2O_2-induced apoptosis/autophagy target expression and changes in the Cx43-interacting protein SGSM3 in H9c2 cells (Figure 11A and 11B). We found that H_2O_2 treatment remarkably increased the expression level

of the Cx43-interacting partner SGSM3, whereas kenpaullone pretreatment significantly down-regulated the level of apoptosis activation (cleaved caspase-3, -9 and cytochrome C) and autophagy target (LC3A/B) expression. These results suggest that kenpaullone protects H9c2 cardiomyocytes from H_2O_2-induced cytotoxicity and apoptosis.

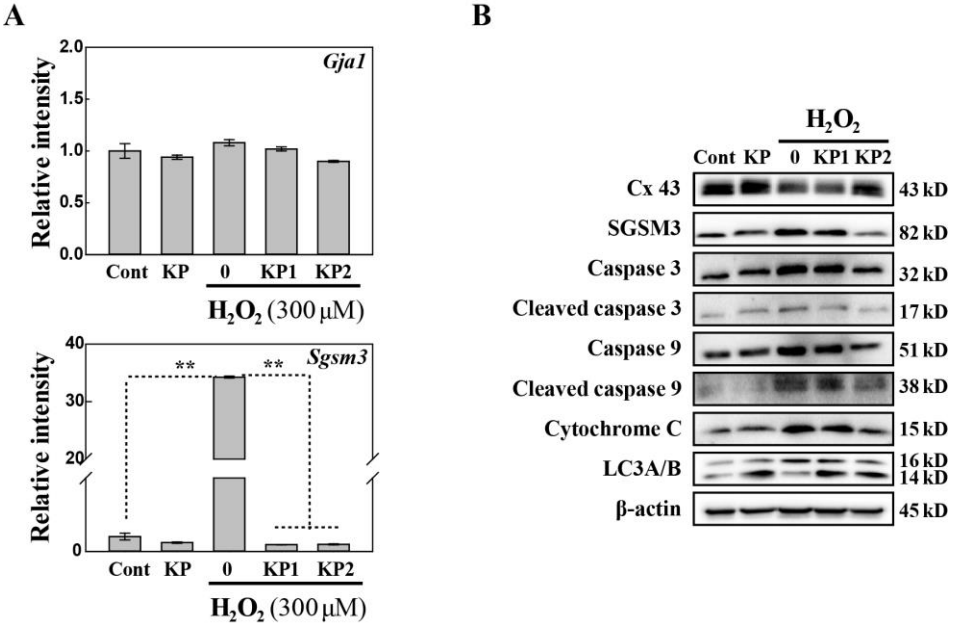

Figure 11. Changes in Cx43-interacting partner SGSM3 and apoptosis/autophagy marker expression in H_2O_2-induced H9c2 cell injury. (A) Quantitative real-time RT-PCR (*Gja1*, *Sgsm3*) and (B) differential

expression of apoptosis and autophagy markers. Relative intensity (%) values of proteins were normalized to beta-actin levels. One-way ANOVA was used to calculate statistical significance between groups, where the *p*-value is $^{*}p<0.05$, $^{**}p<0.01$.

7. Protective effects of kenpaullone on H_2O_2-induced injury in H9c2 cells by modulating Cx43-interacting partner SGSM3 and apoptosis/autophagy marker expression

To assess the function of *Gja1* on H_2O_2-induced injury and kenpaullone pretreatment effects in H9c2 cells, we performed *Gja1/SGSM3* knockdown (KD) experiments for 24 h in H9c2 cells, and cell viability was determined using CCK-8 assays. KD of *Gja1* by siRNA transfection in H9c2 cardiomyocytes prior to H_2O_2-induced injury enhanced SGSM3 expression and significantly decreased cell viability compared with controls (Figure 12A and 12B). Additionally, *SGSM3* KD resulted in an increase in cell viability under H_2O_2-induced injury. Interestingly, kenpaullone treatment in *Gja1* KD H9c2 cells significantly decreased the expression level of SGSM3 and probably protected H_2O_2-induced H9c2 cells from apoptosis/autophagic degradation due

to SGSM3 elevation-induced turnover (Figure 12B). These results indicate that kenpaullone could alleviate H_2O_2-induced apoptosis in H9c2 cells.

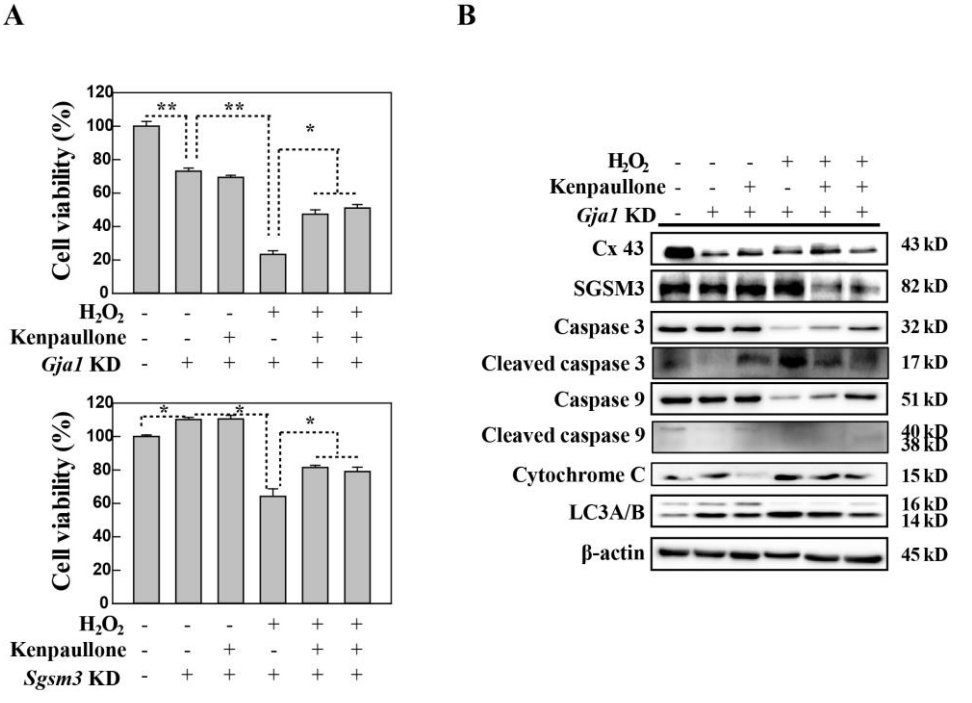

Figure 12. Effects of *Cx43*/*SGSM3* knockdown on the expression level of apoptosis/autophagy proteins and the impact of kenpaullone on H_2O_2-induced H9c2 cell injury. (A) Cell viability and (B) immunoblot analysis. Data are representative of three independent experiments. Significant differences between control and treated groups were determined by ANOVA,

with *p* values indicated as *$p<0.05$ and **$p<0.01$.

8. Changes in Cx43 and SGSM3 expression by kenpaullone pretreatment in H_2O_2-induced H9c2 cells

As shown in Figure 13, the intensity of the localization pattern following multicolor immunofluorescence staining was used to evaluate the relative levels of SGSM3 and Cx43 after kenpaullone treatment in H_2O_2-induced H9c2 cells. Consistent with the previous results, a high SGSM3 expression was observed in H_2O_2-exposed H9c2 cells, while low Cx43 expression was detected in H_2O_2-induced cardiomyocytes. Kenpaullone pretreatment of H_2O_2-induced cells significantly restored Cx43 expression compared with that observed in cells treated with H_2O_2 only. Based on these results, we hypothesized that SGSM3 interacts with Cx43, indicating that SGSM3 plays an important role in gap junctional intercellular communication (GJIC) or cell survival in H_2O_2-induced cardiomyocytes.

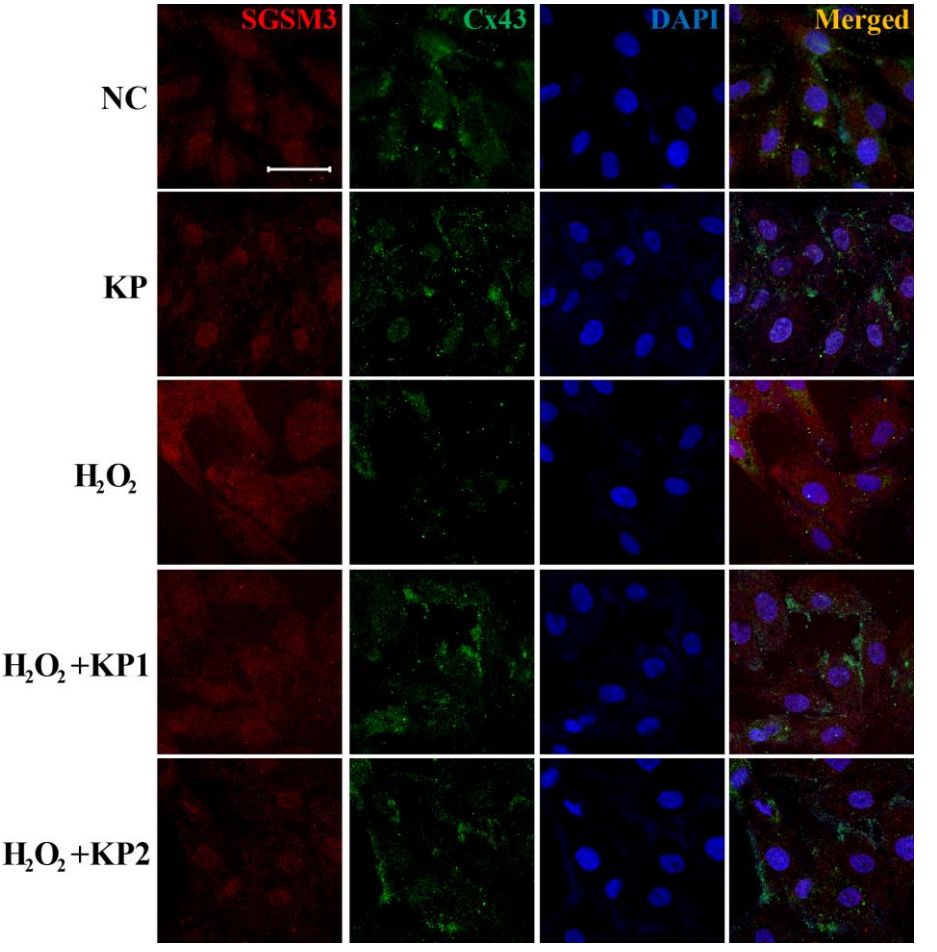

Figure 13. Kenpaullone attenuates H_2O_2-induced injury in H9c2 cells by modulating interactions between Cx43 and SGSM3. Co-localization of Cx43 and SGSM3 proteins by multicolor immunofluorescence. Multicolor labeling with Cx43 (green) and SGSM3 (red) indicates that these markers are co-expressed in H9c2 cells. Nuclei were stained with DAPI (blue). Slides were

observed at 200× magnification, and the scale bar represents 50 μm.

IV. DISCUSSION

In the current study, we investigated potential targets of Cx43 to identify new molecular proteins for cardio-protection. The regulation of Cx43 turnover and degradation by the proteasomal or lysosomal pathway (as part of the autophagic or endocytic pathway) has been well documented in previous studies.[6, 7, 29] Moreover, recent evidence has suggested that Cx43 has additional important effects in regulating cell metabolism by mechanisms independent of cell-to-cell communication.[9-11] However, it is unclear which Cx43 binding proteins are correlated with apoptosis or autophagy in the infarcted heart. Here, we report for the first time that the Cx43-interacting partner SGSM3 affects Cx43 degradation through apoptosis/autophagy in MI of heart.

Recent studies have shown that autophagy is involved in the pathophysiological process of MI, and the cell death mechanisms include not only apoptosis and necrosis but also autophagy in cardiomyocytes.[30] Additionally, autophagy is activated during both the acute and chronic stages of MI.[31] However, previous studies by the Minatoguchi laboratory have established that the enhanced autophagy serves to limit injury to the heart after the infarct.[32] For example,

autophagic inhibition using bafilomycin A1 significantly aggravated post-infarction cardiac dysfunction, whereas autophagic enhancement with rapamycin decreased cardiac dysfunction.[32]

Cardiomyocyte death during I/R causes a loss of heart function. For that reason, many studies have focused on how to protect cardiomyocytes from cytotoxic stimuli such as oxidative stress.[20, 33-36] It has been well documented that oxidative stress is a crucial event in the development of cardiovascular diseases such as atherosclerois, myocardial I/R injury, and heart failure.[37]

In the current study, we investigated the protective effects of kenpaullone on cardiomyocytes following H_2O_2-induced oxidative stress via the interaction of SGSM3 with Cx43. Recently, we reported that the Cx43-interacting partner SGSM3 affects Cx43 degradation through apoptosis/autophagy in the infarcted heart of rats.[8] Moreover, recent evidence has suggested that Cx43 has additional important effects on the regulation of cell metabolism by mechanisms independent of cell-to-cell communication.[9, 11] Here, we report for the first time that the SGSM3 affects Cx43 degradation through apoptosis/autophagy in H_2O_2-induced H9c2 cells, while kenpaullone pretreatment restores Cx43 expression under oxidative stress. Kenpaullone is an inhibitor of glycogen synthase kinase 3β (GSK3β) and down-regulates BCL2/adenovirus E1B 19 kDa

protein-interacting protein 3 (BNIP3), one of the key molecules in cardiac cell death, thereby protecting cardiomyocytes from cell death following ischemic injury.[23]

Alterations in cardiac connexin expression are well established as a consistent feature of heart disease and are associated with life-threatening ventricular arrhythmias. Generally, connexin research has shown that lysosomal degradation pathways modulate connexin proteolysis.[38, 39] Similar to our results, protection of Cx43 from degradation was enhanced by proteasomal and lysosomal inhibitors in brefeldin A-induced Cx43 degradation experiments.[40] Different studies have provided evidence that an 85-kDa Cx43-interacting protein, CIP85 (also known as SGSM3), is a critical factor for Cx43 internalization in GJs from the plasma membrane and for lysosomal degradation.[41, 42] Our findings showed that a Cx43-interacting protein, SGSM3, plays a crucial role in stressed cells. Furthermore, increasing evidence indicates that turnover of Cx43 by SGSM3 overexpression is increased by oxidative stress, which then enhances Cx43 internalization in GJs from the plasma membrane. [40, 41]

V. CONCLUSION

Our findings revealed that kenpaullone protects H9c2 cells against H_2O_2-induced injury by inhibiting Cx43 degradation and that inhibition of the apoptosis pathway is involved in the cardioprotective effect of kenpaullone. Furthermore, our study was the first to suggest that the interaction of SGSM3 with Cx43 may play a key role in Cx43 internalization for connexin turnover in cardiomyocytes of infarcted hearts and oxidative stress-induced cardiomyocyte damage.

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ABSTRACT (IN KOREAN)

Small G protein signaling modulator 3의 허혈성 심근 세포에 대한 영향

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주현철

심장 및 피부조직에서 발현되는 연결 인자 인 Connexin 43 (Cx43)은 다양한 유전적 조건과 관련이 있다고 잘 알려져 있다. 따라서 Cx43 와 상호 작용하는 단백질을 찾아내고 동역학을 이해하는 것은 gap junction 이 매개하는 세포 간 교통의 병적 부전 원리뿐만 아니라 Cx43 의 알려지지 않은 생물학적 기능을 확인하는 데 중요하다. 본 연구에서는 심장 보호에서 관여하는 새로운 분자 기능을 결정하는 Cx43 의 잠재적 표적을 관찰했다. 공면역 단백 침전법(co-immunoprecipitated)을 통한 MALDI-TOF

질량 분석결과 Cx 43 과 상호작용하는 몇몇 단백질을 찾았다. GeneMANIA 네트워크 분석에서, 이전에 Cx43 과 관련성이 밝혀지지 않은 SGSM3 가 심장 기능에서 Cx43 와 높은 상관 관계가 있었으며 SGSM3 의 증가는 심근 경색된 쥐 심근 세포에서 리소좀 분해를 통한 Cx43 의 분해를 유도하는 것으로 나타났다. Cx43 의 분해는 H9c2 심근 세포에서도 SGSM3 와 Cx43 사이의 상호 작용에 의존하는 것을 확인했다. 또한 H_2O_2 에 의해 유도된 산화 스트레스 조건에서 SGSM3 와 Cx43 의 상호 작용과 관련하여 kenpaullone 의 보호 효과도 조사했다. 먼저 H9c2 세포에서 Cx 43 이 H2O2 농도에 따라 유의하게 하향 조절되는 반면, SGSM3 의 발현은 H_2O_2 농도에 따라 상향 조절된다는 것을 발견했다. H_2O 유도 된 세포 독성에 kenpaullone 전처리의 영향을 H9c2 세포에서 평가하였다. H_2O_2 는 lactate dehydrogenase (LDH)의 방출을 현저히 증가 시켰고, kenpaullone 전처리는 H9c2 세포에서 LDH 방출을 억제했다. SGSM3 과 Cx43 사이의 상호 작용의 기능적 중요성은 Cx43 발현이 H9c2 세포에서 SGSM3 siRNA knock down 에 의해 강화되는 결과로 확인되었다. 또한 kenpaullone 전처리는 apoptosis 활성화 유전자 (cleaved caspase-3, cleaved caspase-9 및 cytochrome C), autophagy marker(LC3A / B) 및

Cx43 상호 작용 파트너인 SGSM3 를 의미있게 억제하였다. 결론적으로, SGSM3 는 심근경색 모델에서 Cx43 의 상호작용 파트너로 확인되었으며 향후 새로운 치료 표적의 수립에 기여할 것으로 보인다. 또한 kenpaullone 은 SGSM3 에 의한 Cx43 분해에 관여하여 산화 스트레스 심근 세포로부터 보호하는 역할을 한다.

핵심 단어: 심근 세포, connexin 43, SGSM3, kenpaullone