Role of connexin-43 in protective PI3K-Akt-GSK-3β signaling in cardiomyocytes

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Ishikawa S, Kuno A, Tanno M, Miki T, Kouzu H, Itoh T, Sato T, Sunaga D, Murase H, Miura T. Role of connexin-43 in protective PI3K-Akt-GSK-3β signaling in cardiomyocytes. Am J Physiol Heart Circ Physiol 302: H2536–H2544, 2012. First published April 13, 2012; doi:10.1152/ajpheart.00940.2011.—Sarcolemmal connexin-43 (Cx43) and mitochondrial Cx43 play distinct roles: formation of gap junctions and production of reactive oxygen species (ROS) for redox signaling. In this study, we examined the hypothesis that Cx43 contributes to activation of a major cytoprotective signal pathway, phosphoinositide 3-kinase (PI3K)-Akt-glycogen synthase kinase-3β (GSK-3β) signaling, in cardiomyocytes. A δ-opioid receptor agonist {[D-Ala²,D-Leu⁵]enkephalin acetate (DADLE)}, endothelin-1 (ET-1), and insulin-like growth factor-1 (IGF-1) induced phosphorylation of Akt and GSK-3β in H9c2 cardiomyocytes. Reduction of Cx43 protein to 20% of the normal level by Cx43 small interfering RNA abolished phosphorylation of Akt and GSK-3β induced by DADLE or ET-1 but not that induced by IGF-1. DADLE and IGF-1 protected H9c2 cells from necrosis after treatment with H₂O₂ or antimycin A. The protection by DADLE or ET-1, but not that by IGF-1, was lost by reduction of Cx43 protein expression. In contrast to Akt and GSK-3β, PKC-ε, ERK, and p58 mitogen-activated protein kinase were phosphorylated by ET-1 in Cx43-knockdown cells. Like diazoxide, an activator of the mitochondrial ATP-sensitive K⁺ channel, DADLE and ET-1 induced significant ROS production in cardiomyocytes, although such an effect was not observed for IGF-1. Cx43 knockdown did not attenuate the mitochondrial ROS production by DADLE or ET-1. Cx43 was coimmunoprecipitated with the β-subunit of G protein (Gb), and knockdown of Gβ mimicked the effect of Cx43 knockdown on ET-1-induced phosphorylation of Akt and GSK-3β. These results suggest that Cx43 contributes to activation of class Iα PI3K in PI3K-Akt-GSK-3β signaling possibly as a cofactor of Gβ in cardiomyocytes. G-protein-coupled receptor; insulin-like growth factor-1; reactive oxygen species; phosphoinositide 3-kinase; glycogen synthase kinase-3β.

CONNEXIN-43 (Cx43) IS A MAJOR SUBUNIT PROTEIN OF GAP JUNCTIONS IN THE VENTRICULAR MYOCARDIUM, AND ITS PHOSPHORYLATION AT SER OR TYR RESIDUES BY PROTEIN KINASES IS ONE OF REGULATORY MECHANISMS OF GAP JUNCTION PERMEABILITY (20, 34). THE NUMBER OF GAP JUNCTIONS AND CONDUCTANCE OF EACH GAP JUNCTION DETERMINE ELECTRICAL COUPLING OF CARDIOMYOCYTES IN THE HEART. HOWEVER, IN ADDITION TO ITS ROLE AS A SUBUNIT OF GAP JUNCTIONS, CX43 PLAYS VARIOUS ROLES IN CELL SURVIVAL AND DEATH, DEPENDING ON CIRCUMSTANCES AND CELL TYPES (9, 20). AN IMPORTANT ROLE OF CX43 IN PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS) IN MITOCHONDRIA FOR REDOX SIGNALING HAS BEEN DEMONSTRATED BY A SERIES OF EARLIER STUDIES (4, 15, 18, 30). IN ADDITION, CX43 HEMICHANNELS IN THE CELL MEMBRANE WERE RECENTLY SUGGESTED TO BE INVOLVED IN SIGNAL TRANSDUCTION BY RECEPTORS AND SIGNALING MOLECULES SUCH AS THE P2Y1 RECEPTOR AND AGS8 (ACTIVATOR OF G-PROTEIN SIGNALING) (8; Refs. 12, 32, 33).

OF SIGNALING PATHWAYS FUNCTIONING FOR CYTOPROTECTION, PHOSPHOINOSITIDE 3-KINASE (PI3K)-Akt-glycogen synthase kinase-3β (GSK-3β) SIGNALING IS A MAJOR ONE, BEING INVOLVED IN PROTECTION OF DIFFERENT TYPES OF CELLS FROM NECROSIS AND APOPTOSIS (19, 21). IN THE MYOCARDIUM, THIS SIGNALING PLAYS A CRUCIAL ROLE IN ANTI-INFARCT TOLERANCE ADOPTED BY INSULIN, ERETHYROPOIETIN, BRAKYNIN, ADENOSINE RECEPTOR AGONISTS, PRECONDITIONING, AND ALSO POSTCONDITIONING (6, 11, 14, 22, 27, 28, 38, 40). STUDIES BY OLDENBURG ET AL. (24, 25) AND BY KRIEG ET AL. (17) INDICATED THAT PI3K-Akt signaling activates production of reactive oxygen species (ROS) BY OPENING OF THE MITOCHONDRIAL ATP-SENSITIVE K⁺ CHANNEL (mKᵣ₅ channel) AND THE FUNCTION OF THE mKᵣ₅ channel REQUIRES A CERTAIN LEVEL OF CX43 PROTEIN IN MITOCHONDRIA (15, 30). HOWEVER, THE ROLE OF CX43 IN PI3K-Akt-GSK-3β SIGNALING HAS NOT BEEN SYSTEMATICALLY EXAMINED.

IN THE PRESENT STUDY, WE EXAMINED WHETHER CX43 PLAYS AN INDISPENSABLE ROLE IN THE PI3K-Akt-GSK-3β SIGNAL PATHWAY IN CARDIOMYO CYTES AND WHETHER ITS ROLE, IF ANY, IS PLAYED BY MITOCHONDRIAL CX43 OR SARCOLEMNAL CX43. SINCE BOTH CLASS Iα AND CLASS Iβ PI3Ks ARE COUPLED WITH Akt-GSK-3β SIGNALING IN CARDIOMYO CYTES, WE COMPARED THE EFFECTS OF STIMULATION OF A CYTOKINE RECEPTOR, INSULIN-LIKE GROWTH FACTOR-1 (IGF-1) RECEPTOR, WITH THE EFFECTS OF STIMULATION OF G-PROTEIN-COUPLED RECEPTORS (GPCRS). THE FUNCTION OF MITOCHONDRIAL CX43 WAS ASSESSED BY MONITORING MITOCHONDRIAL ROS PRODUCTION USING MITO TRACKER RED FLUORESCENCE AS AN INDICATOR. INVOLVEMENT OF CX43 IN THE SIGNAL TRANSDUCTION WAS EXAMINED BY KNOCKING DOWN CX43 PROTEIN EXPRESSION AND BY IMMUNOPRECIPITATION OF CX43 WITH SIGNALING MOLECULES. RESULTS OF THE EXPERIMENTS INDICATED THAT CX43 CONTRIBUTES TO ACTIVATION OF Akt-GSK-3β SIGNALING BY CLASS Iα PI3K POSSIBLY VIA ITS INTERACTION WITH THE β-SUBUNIT OF G-PROTEIN (Gb) BUT NOT VIA PRODUCTION OF MITOCHONDRIAL ROS.

METHODS

THIS STUDY WAS APPROVED BY THE ANIMAL RESEARCH ETHICS COMMITTEE OF SAPPORO MEDICAL UNIVERSITY. CELL CULTURE. H9c2 cells (rat cardiomyoblast cell line) and A7r5 cells (rat aortic smooth muscle cell line) were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS at 37°C with 5% CO₂. THE CELLS WERE USED FOR EXPERIMENTS WHEN THEY WERE 70-80% CONFLUENT, AND FBS WAS REMOVED FROM THE MEDIUM 24 H BEFORE PHARMACOLOGICAL TREATMENTS AND/OR INDUCTION OF CELL DEATH.

ACTIVATION OF GPCRS AND IGF-1 RECEPTOR. TO ACTIVATE PI3K-Akt-GSK-3β SIGNALING, H9c2 cells were treated with 300 nM [D-Ala², D-Leu⁵]enkephalin acetate (DADLE), a δ-opioid receptor agonist; 10 nM endothelin-1 (ET-1); or 10 nM IGF-1 for 5 min before preparation

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of cell lysates. The doses of these receptor agonists were selected on the basis of results of preliminary experiments using 300–1,000 nM DADLE, 1–100 nM ET-1, and 1–10 nM IGF-1 and results of our previous studies (20, 23). Cells that received vehicles served as controls.

Immunoblotting and immunoprecipitation. Whole cell lysates of H9c2 cells were prepared by using CellLytic-M mammalian cell lysis/extraction agent (Sigma). In some experiments, mitochondrial and cytosolic fractions or sarcolemna-rich fractions and cytosolic fractions were separated by a mitochondrial isolation kit (Pierce Biotechnology, Rockford, IL) or by a plasma membrane protein extraction kit (Abcam, Cambridge, MA), respectively. The samples were electrophoresed in 12.5% SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Proteins were visualized by use of antibodies against each protein of interest and Immobilon (Millipore, Bedford, MA). Protein levels were determined by a lumino-image analyzer (LAS-3000; Fujifilm, Tokyo, Japan). As primary antibodies for immunoblotting, antibodies against Akt, GSK-3β, extracellular signal-regulated protein kinase (ERK), Ser473-phospho-Akt, Ser9-phospho-GSK-3β, Thr202/Tyr204-phospho-ERK, protein kinase Cε (PKC-ε), p38 mitogen-activated protein kinase (p38 MAP kinase), Thr180/Tyr182-phospho-p38 MAP kinase (Cell Signaling Technology, Beverly, MA), Ser729-phospho-PKC-ε (Upstate, Lake Placid, NY), Gβ, GAPDH, Na+–K+–ATPase (Santa Cruz Biotechnology, Santa Cruz, CA); p110y subunit of PI3K (#5405, Cell Signaling; sc-7177, Santa Cruz Biotechnology), Cx43 (C6219; Sigma), Cx40 and Cx45 (nos. AB1726 and AB1745; Millipore, Billerica, MA) were used. For immunoprecipitation of Cx43, antibody no. 610062 (BD Biosciences, Sparks, MD) was used. Immunoprecipitation of Cx43, Gβ, or p110y was performed using 400 μg proteins as previously reported (20).

Suppression of protein expression by small interfering RNA methods. H9c2 cells were transfected with small interfering (si)RNAs using the Cell Line Nucleofector Kit L (Amaxa, Gaithersburg, MD) 48 h before experiments in which responses of signaling pathways were examined by use of receptor agonists. The sense and antisense siRNAs in the cocktail were as follows: CAACCAAA-3′, extracellular signal-regulated protein kinase (ERK), Ser473-phospho-Akt, Ser9-phospho-GSK-3β, Thr202/Tyr204-phospho-ERK, protein kinase Cε (PKC-ε), p38 mitogen-activated protein kinase (p38 MAP kinase), Thr180/Tyr182-phospho-p38 MAP kinase (Cell Signaling Technology, Beverly, MA), Ser729-phospho-PKC-ε (Upstate, Lake Placid, NY), Gβ, GAPDH, Na+–K+–ATPase (Santa Cruz Biotechnology, Santa Cruz, CA); p110y subunit of PI3K (#5405, Cell Signaling; sc-7177, Santa Cruz Biotechnology), Cx43 (C6219; Sigma), Cx40 and Cx45 (nos. AB1726 and AB1745; Millipore, Billerica, MA) were used. For immunoprecipitation of Cx43, antibody no. 610062 (BD Biosciences, Sparks, MD) was used. Immunoprecipitation of Cx43, Gβ, or p110y was performed using 400 μg proteins as previously reported (20).

Quantitative real-time RT-PCR. Cells were lysed and mRNA was extracted by using an RNeasy Mini kit (Qiagen, Germantown, MD) according to the manufacturer’s instructions. First-strand cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA). Quantitative real-time RT-PCR analysis was performed for determination of mRNA levels of Cx45 with a StepOne (Applied Biosystems, Foster City, CA) using the Power SYBR Green Mater Mix. The following primers were used: for Cx43, 5′-AAAGACGAGCGAC-3′ and 5′-CTCCACCCCTCAACACAGTCCT-3′; and for GAPDH, 5′-TCACCACCATGGAGAAGGC-3′ and 5′-GCTAAGCAGTTGGCGTGGCA-3′. The thermal cycler conditions were as follows: hold for 10 min at 95°C and then by two-step PCR for 40 cycles of 95°C for 15 s followed by 60°C for 1 min. All assays were performed in duplicate. Differences between mRNA levels in study groups were examined by the comparative Ct method using endogenous GAPDH as an internal control.

Immunocytochemistry. Cells were cultured on collagen-coated glass dishes and were stained with 0.2 μM Mito-Tracker Red for 15 min. Stained cells were then fixed with 4% paraformaldehyde, washed with PBS, blocked with 3% BSA in PBS for 30 min, and incubated in PBS containing 3% BSA and anti-Cx43 antibodies.

Determination of mitochondrial ROS production. The level of mitochondrial ROS production in H9c2 cells was determined by using MitoTracker Red as previously reported (17, 23). H9c2 cells were treated with a vehicle, 300 nM DADLE, 10 nM ET-1, or 100 μM diazoxide (an mKATP channel opener) and 1 μM MitoTracker Red for 15 min. Cells were then washed with the medium without MitoTracker Red. With the use of a fluorescent microscope, level of MitoTracker Red fluorescence of ≥40 cells in each well was determined and then averaged for each experiment. Control cells (i.e., control siRNA-transfected and vehicle-treated cells) were always included in each culture plate, and the fluorescence level in control cells was used to normalize values in the other treatment groups in the same plate.

Induction of cell necrosis. H9c2 cells were incubated with 100 μM H2O2 or 60 μM antimycin A for 3 h. Doses of the two agents were selected to induce different levels of necrosis (see RESULTS). One hour before addition of H2O2 or antimycin A, cells were pretreated with DADLE, ET-1, IGF-1, or vehicle. At the end of the experiments, cells were fixed and stained with propidium iodide. More than 400 cells in each well were counted, and the percentage of cells with a propidium iodide-positive nucleus was determined as an index of cell necrosis.
Blockade of Cx43 hemichannels and gap junctions. To examine the effects of blockade of Cx43 hemichannels and gap junctions on protein kinase activation by GPCRs, H9c2 cells were incubated with an inhibitor of Cx43 channels, GAP27 (Sigma; Ref. 8), for 1 h before treatment with ET-1 or its vehicle. Cell lysates were prepared at 5 min after the treatment for immoblotting.

Statistical analysis. Results are presented as means ± SE. Differences between groups were tested by one-way or two-way ANOVA, and the Student-Newman-Keuls post hoc test was used for multiple comparisons when ANOVA indicated significant differences. Differences were considered significant when \( P < 0.05 \).

RESULTS

Effects of siRNA knockdown of Cx43 and Gβ on PI3K-Akt-GSK-3β signaling. Transfection of Cx43 siRNA reduced Cx43 protein by ~80%, and Cx43 levels in the mitochondrial and cytosolic fractions were similarly reduced (Fig. 1A). Co-staining of mitochondria and Cx43 confirmed that Cx43 siRNA suppressed Cx43 expression in mitochondria and other compartments in the cell (Fig. 1B). Significant phosphorylation of Akt and GSK-3β was induced by DADLE, ET-1, or IGF-1 as shown in Figs. 2 and 3. Cx43 knockdown attenuated Akt- and GSK-3β phosphorylation after treatment with DADLE or ET-1 (Fig. 2). However, phosphorylation of the kinases in response to IGF-1 was preserved (Fig. 3, A and B). Inhibition of Jak2 by AG490 inhibited Akt phosphorylation induced by IGF-1 (Fig. 3C). In contrast to Akt phosphorylation induced by DADLE and ET-1 (Fig. 2), IGF-1-induced phosphorylation of Akt was accompanied by reduction of total Akt protein level (Fig. 3, A and C).

In whole lysates of untreated H9c2 cells, Cx43 was co-immunoprecipitated with Gβ (Fig. 4A), although interaction of
Cx43 with p110γ of PI3K could not be examined since the anti-p110γ we used did not work for immunoprecipitation in our hands (data not shown). To confirm interaction of Cx43 with Gβ in the membrane, we prepared sarcolemma-rich fractions and immunoprecipitated Gβ. As shown in Fig. 4B, the Cx43 signal was detected in Gβ immunoprecipitates from sarcolemma-rich fractions. Similar to Cx43 knockdown, knockdown of Gβ (Gβ1 and Gβ2) abolished phosphorylation of Akt and GSK-3β by ET-1 (Fig. 4, C and D). However, phosphorylation of these kinases by IGF-1 was not lost by Gβ knockdown (data not shown).

To examine whether the inhibitory effect of Cx43 knockdown on PI3K-Akt-GSK-3β signaling is specific to this signaling pathway, effects of Cx43 knockdown on responses of PKC-ε, ERK, and p38 MAP kinase were assessed. As shown in Fig. 5, PKC-ε, ERK, and p38 MAP kinase were similarly phosphorylated by ET-1 in control and Cx43 siRNA-transfected cells. Gβ siRNA also failed to inhibit p38 MAP kinase phosphorylation by ET-1 (Fig. 5B).

In A7r5 cells, a smooth muscle cell line, phosphorylation of Akt and that of GSK-3β by ET-1 was not affected by reduction of Cx43 expression (Fig. 6). These findings suggest that the role of Cx43 in GPCR-induced PI3K-Akt-GSK-3β signaling is specific to cardiomyocytes.

Effects of Cx43 knockdown on mitochondrial ROS production. As in previous studies on rabbit cardiac mitochondria (17, 24, 25), mitochondria in H9c2 cells generated ROS in response to treatment with DADLE or ET-1 (Fig. 7). However, IGF-1 did not induce mitochondrial ROS production. In contrast to genetic partial deletion of Cx43 in mouse myocytes (15), Cx43 knockdown using siRNA did not reduce mitochondrial ROS production by activation of the mKATP channel. By blotting for Cx43 in samples separated from those for ROS determination (n/1005), we confirmed that Cx43 siRNA reduced Cx43 to 18% of controls in this series of experiments as well. ROS production in response to DADLE and ET-1 treatment was also unaffected by Cx43 knockdown.

Effects of Cx43 knockdown on cytoprotection afforded by PI3K-Akt-GSK-3β signaling. Exposure to 100 μM H2O2 for 3 h induced necrosis in 30–40% of control siRNA-transfected cells. Pretreatment with DADLE and that with IGF-1 significantly reduced cell necrosis to 43 and 58% of the control, respectively (Fig. 8). Suppression of Cx43 expression by its siRNA abolished the protection afforded by DADLE but not the protection afforded by IGF-1. Incubation with 60 μM antimycin A for 3 h induced more extensive injury than did the H2O2 treatment, resulting in necrosis in almost all cells in the control groups. Pretreatment with ET-1 and that with IGF-1 afforded comparable protection, reducing cell necrosis by 23 and 33%, respectively. A protective effect of ET-1 was not detected in cells in which Cx43 protein was reduced by siRNA.
though the protection by IGF-1 was unaffected by Cx43 siRNA (Fig. 9).

Effects of Cx40 and Cx45 knockdown on PI3K-Akt-GSK-3β signaling. Since Cx43 knockdown inhibited activation of PI3K-Akt-GSK-3β signaling by ET-1 (Fig. 2), we performed a post hoc series of experiments to examine the involvement of Cx40 and Cx45 in the signaling. Cx40 siRNA reduced Cx40 protein level by 66%, but it did not inhibit phosphorylation of Akt induced by ET-1 (Fig. 10, A and B). Since detection of Cx45 in H9c2 cells by a commercial anti-Cx45 antibody was unsuccessful, we confirmed the efficacy of Cx45 siRNA by quantitative RT-PCR (Fig. 10C). Pretreatment with Cx45 siRNA inhibited ET-1-induced phosphorylation of Akt (Fig. 10D).

Effects of a gap junction blocker on Cx43-mediated Akt signaling. To examine whether the role of Cx43 in PI3K activation depends on open status of the Cx43 channel (i.e., hemichannel and/or gap junctions), we used 37,43GAP27, a peptide mimetic of the extracellular loop of Cx43 and Cx37. As shown in Fig. 11, phosphorylation of Akt by ET-1 was inhibited by pretreatment with 37,43GAP27.

DISCUSSION

In the heart, two classes of PI3K, class IA and class IB, are responsible for Akt activation, and these classes of PI3K are coupled with different types of receptors. Cytokine receptors
activate class IB PI3K via G
/G9252
GSK-3
H9c2 cells, A7r5 cells showed similar responses of Akt and
not impair all signal transmission from GPCRs. In contrast to
Cx43 did not inhibit activation of PKC-
, ERK, and p38 MAP
ε
receptor remained unchanged. Importantly, knockdown of
responses of the same kinases to activation of the IGF-1
/H9254
-opioid receptor and activation of the ET receptor, whereas
/H9252
and GSK-3
showed that
/H9253
suggest that Cx43 is involved in activation of class IB PI3K by
of Cx43 expression (Fig. 6). Taken together, these findings
class IB PI3K, we postulated two possibilities: promotion of
mKATP-mediated ROS generation and enhancement of p110
G
/P9252/H9253
-PI3K-Akt-GSK-3
signaling (Fig. 4
C
). Knockdown of the G
/H9252
subunit-mediated interaction of
interactions because commercially available anti-
plex of noncontiguous lanes in the same
recently showed that diazoxide-induced K+
channel activity, leading to increased ROS production (15, 18,
30). In a study by Heinz et al. (15) using MitoTracker to
determine mitochondrial ROS, production of ROS in response to
treatment with diazoxide, an mKATP channel opener, was
significantly reduced in myocytes isolated from heterozygous
Cx43-knockout mice. Furthermore, Rottlaender et al. (30)
recently showed that diazoxide-induced K+
clamping of the inner membrane of mitochondria was
significantly attenuated in mitochondria of heterozygous Cx43-
knockout mice. We first assumed that ROS generated by
mitochondria may activate Ras, an activator of PI3K, and PKC,
an upstream kinase of GSK-3β. However, mitochondrial ROS
produced by diazoxide, DADLE, or ET-1 was unchanged by
Cx43 siRNA, which reduced Cx43 by ~80% (Fig. 7). The
reason for the discrepancy between the present findings and
those by Heizel et al. (15) is unclear. However, the difference
in species (rat vs. mouse) or other experimental conditions
might be responsible. Nevertheless, the present results indicate
that Cx43 contributes to PI3K activation by a mechanism other
than promotion of mitochondrial ROS production.

To the best of our knowledge, no evidence for possible involve-
ment of Cx43 in p110γ-Gβγ interactions has been reported.
However, Cx43 in the cell membrane has been reported to interact
with various signaling molecules, including P2Y1 receptor and
AGS8 (12, 32–34). In the present experiments, Cx43 was coim-
munoprecipitated with G
/H9252
and Gβγ-3β to activation of the ET receptor regardless of the level
of Cx43 expression (Fig. 6). Taken together, these findings
suggest that Cx43 is involved in activation of class IIβ PI3K by
GPCRs characteristically in cardiomyocytes, although Cx43 is
unnecessary for class IA PI3K activation.

As a mechanism by which Cx43 contributes to activation of
class IIβ PI3K, we postulated two possibilities: promotion of
mKATP-mediated ROS generation and enhancement of p110γ-
Gβγ interactions. Cx43 localizes in the inner membrane of
subsarcolemmal mitochondria (4), and it augments mKATP
channel activity, leading to increased ROS production (15, 18,
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recently showed that diazoxide-induced K+
channel activity, leading to increased ROS production (15, 18,
In a post hoc series of experiments, we examined whether the role of Cx43 in activation of class IB PI3K is shared by Cx40 and Cx45. Knockdown of Cx40 protein expression did not inhibit Akt phosphorylation by ET-1, but Cx45 knockdown mimicked the effects of Cx43 knockdown on Akt phosphorylation. Interestingly, a recent study (29) has shown that Cx43 forms gap junction plaque with Cx45 but not with Cx40 in HeLa cells, indicating functional intimacy of Cx43 and Cx45. However, Cx45 is expressed at the early stage of heart development and is downregulated after birth (2, 3). Thus the present finding regarding Cx45 in H9c2 cells may not be extrapolated to adult hearts in situ.

In another post hoc series of experiments, 37,43GAP27, a peptide inhibitor of Cx43 and Cx37 (8), blocked response of Akt to activation of the ET receptor (Fig. 11). Since Cx37 is expressed in endothelial cells but not in cardiomyocytes, the results shown in Fig. 11 suggest that either transport of certain molecules through the Cx43 channel or configuration change in the channel per se contributes to activation of class IB PI3K after GPCR stimulation. However, the present data are preliminary, and the relationship between functional status of the Cx43 channel and class IB PI3K remains unclear.

The present observations carry some clinical implications. Cardiac tissue samples from heart failure patients and animal models of heart failure showed reduction, redistribution, and/or increased heterogeneity of Cx43 expression in cardiomyocytes (1, 7, 16, 35). These changes in Cx43 expression have been discussed as possible underlying mechanisms of ventricular arrhythmias and dyssynchrony (5, 39). However, the present results raise the possibility that some of the known modifications of signaling pathways downstream of GPCRs in failing hearts (10, 26) are attributable to insufficient level of Cx43 in the sarcolemma. Nevertheless, roles of the Cx43 hemichannel in signal transduction and its interaction with signaling molecules may warrant further investigation.

There are limitations in this study. We cannot exclude the possibility that Cx43 in mitochondria contributes to class IB PI3K activation by a mechanism independent of ROS production. However, no signaling molecule other than ROS has been shown to be regulated by Cx43 in mitochondrial inner membranes. To examine the involvement of mitochondrial Cx43 in PI3K activation, we attempted to inhibit recruitment of cytosolic Cx43 to mitochondria by using geldanamycin, an HSP90

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Fig. 7. Mitochondrial reactive oxygen species production in H9c2 cells. Level of Mito Tracker Red fluorescence after each treatment was normalized by average level in the control siRNA-treated group. *P < 0.05 vs. untreated control in control siRNA-treated groups. †P < 0.05 vs. untreated control in Cx43 siRNA-treated groups.

Fig. 8. Effects of DADLE and IGF-1 on myocardial necrosis induced by hydrogen peroxide. Necrosis of H9c2 cells was induced by treatment with 100 μM hydrogen peroxide for 3 h. DADLE and IGF-1 were added to the culture medium 1 h before treatment with hydrogen peroxide. *P < 0.05.
inhibitor. Unfortunately, our attempt was unsuccessful since treatment of H9c2 cells with 0.1 μM geldanamycin for 4–24 h failed to significantly reduce Cx43 in the mitochondrial fraction without cytotoxicity. It is also unclear whether Cx43-PI3K interaction is modified by phosphorylation of Cx43 at Ser and/or Tyr residues.

In conclusion, the present study suggests that Cx43 is required for activation of class Iβ PI3K by GPCRs, possibly as a cofactor of Gβ, leading to Akt-GSK-3β-mediated protection against necrosis in cardiomyocytes.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**Fig. 9.** Effects of ET-1 and IGF-1 on cell necrosis induced by antimycin A (AA). Necrosis of H9c2 cells was induced by treatment with 60 μM antimycin A for 3 h. ET-1 and IGF-1 were added to the culture medium 1 h before treatment with antimycin A. *P < 0.05.

**Fig. 10.** Effects of Cx40 and Cx45 knockdown on Akt- and GSK-3β phosphorylation in H9c2 cells. Knockdown of Cx40 protein (A) did not inhibit phosphorylation of Akt and GSK-3β in response to ET-1 treatment (B). In contrast, knockdown of Cx45 (C) inhibited ET-1-induced phosphorylation of Akt and GSK-3β (D).

**Fig. 11.** Effects of GAP27 on Akt and GSK-3β phosphorylation in H9c2 cells. Pretreatment with 37,43GAP27 for 1 h attenuated phosphorylation of both Akt and GSK-3β by ET-1. Responses of the kinases 5 min after treatment with ET-1 are shown.

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