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Cholesterol modulates function of connexin 43 gap junction channel via PKC pathway in H9c2 cells

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ABSTRACT

It has been shown that cholesterol modulates activity of protein kinase C (PKC), and PKC phosphorylates connexin 43 (Cx43) to regulate its function, respectively. However, it is not known whether cholesterol modulates function of Cx43 through regulating activity of PKC. In the present study, we demonstrated that cholesterol enrichment reduced the dye transfer ability of Cx43 in cultured H9c2 cells. Western blot analysis indicated that cholesterol enrichment enhanced the phosphorylated state of Cx43. Immunofluorescent images showed that cholesterol enrichment made the Cx43 distribution from condensed to diffused manner in the interface between the cells. In cholesterol enriched cells, PKC antagonists partially restored the dye transfer ability among the cells, downregulated the phosphorylation of Cx43 and redistributed Cx43 from the diffused manner to the condensed manner in the cell interface. In addition, reduction of cholesterol level suppressed PKC activity to phosphorylate Cx43 and restored Cx43 function in PKC agonist-treated cells. Furthermore, we demonstrated that cholesterol enrichment upregulated the phosphorylated state of Cx43 at Ser368, while PKC antagonists reversed the effect. Taken together, cholesterol level in the cells plays important roles in regulating Cx43 function through activation of the PKC signaling pathway.

1. Introduction

Connexins assemble to form gap junction channels to mediate communication and signaling between adjacent cells by allowing the passage of ions, metabolites and signaling molecules. Connexins interact with caveolins and partition into lipid raft domains [1] such that membrane cholesterol plays a significant role in regulating function of gap junction channels [2,3]. Connexin43 (Cx43) is predominantly expressed in the heart where it supports the spread of the action potential that ensures the coordinated contractile activation of the heart. The normal heart rhythm thus depends fundamentally on the coupling of gap junctions in cardiomyocytes. In cholesterol-fed rabbits, elevated cholesterol level results in redistribution of Cx43 gap junctions at the lateral membrane of cardiomyocytes, suggesting that Cx43 is involved in the molecular mechanism of hypercholesterolemia-induced cardiac contractile dysfunction and dysrhythmias [4]. Recently, it has been indicated that loss of cardioprotection by ischemic preconditioning in cholesterol dieted rats is associated with a redistribution of both sarcolemmal and mitochondrial Cx43 [5]. These pieces of evidence imply that cholesterol might regulate the function of the Cx43 gap junction channel.

Activation of protein kinase C (PKC) can lead to phosphorylation of Cx43, which affects trafficking, assembly, degradation, and channel gating of Cx43 gap junction channels [6–8]. On the other hand, cholesterol induces activation of PKC in cultured ascites tumor cells [9] and cholesterol-fed rabbits exhibited an increase in PKC activity in smooth muscle cells [10]. In addition, it has been demonstrated that cholesterol can amplify the activity of PKC in the presence of diacylglycerol [11]. Therefore, we hypothesize that cholesterol can regulate the function of Cx43 through a PKC dependent pathway. In the present study, using methly-β-cyclodextrin (MβCD) to manipulate membrane cholesterol content, we studied the effect of cholesterol on the function and distribution of Cx43 in H9c2 cell line, a standard cardiac cell line derived from embryonic cells. Our results demonstrated that cholesterol plays an important role in modulating the function of the Cx43 gap junction channel through a PKC-dependent pathway.

2. Materials and methods

2.1. Materials

Staurosporine (STS), filipin, DAPI, MβCD, cholesterol loaded MβCD (MβCD/Chol) and rabbit anti-Cx43 antibody were from Sigma-Aldrich.

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PBS, FBS and HBSS were from Gibco. PMA solution was from MultiSciences Biotech. Calphostin C was from Merck Biosciences. Lucifer yellow was from Molecular probes. Phospho-Connexin 43 (Ser368) was from Cell Signaling.

2.2. Cell culture

Rat embryonic ventricular cell line H9c2 (Chinese Academy of Sciences Cell Bank, Shanghai) was cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 mg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2–3 days and the cells were subcultured regularly. For experiments, cells were cultured on 6-well plates, kept in DMEM with 10% FBS until 80%–90% confluence.

2.3. Western blotting

The cultured H9c2 cells were homogenized on ice in lysis buffer (8 mol/L urea, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 50 mmol/L Tris–HCl, pH = 8.0, supplemented with a proteinase inhibitor cocktail (Sigma) and sonicated three times for 10 s on ice. The protein in the samples was quantified by Bradford's method. Western blotting was performed as described previously [12]. A 10 μg sample of each protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel and the band was transferred to the PVDF membrane (Millipore). The membranes were preincubated with 5% skim milk in PBS containing 0.05% Tween 20 for 1 h, then incubated with 1:10,000 anti-connexin43 (Sigma) and 1:1000 anti-GAPDH (Epitomics) antibody overnight. Afterwards the membranes were washed in PBS containing 0.05% Tween 20, followed by incubation with anti-rabbit immunoglobulin-conjugated peroxidaselabeled dextran polymer (1:1000, Jackson) for 1 h at room temperature. The immunoreactivity was visualized by use of an ECL plus western blotting detection system (Millipore). Densitometric analyses were performed with Adobe photoshop7.0.1 software package.

2.4. Immunofluorescence analysis

The H9c2 cells cultured on six-well glass chamber slides were fixed in 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and stained with antibodies. For immunofluorescence analysis, rabbit anti-Cx43 antibody (1:400) was applied as primary antibodies. The sections were blocked with 5% goat serum, incubated with antibodies diluted in blocking reagent overnight at 4 °C. Afterwards, the sections were washed 3 times in TBST buffer and incubated with DyLight549 goat anti-rabbit immunoglobulin (Jackson) for 1 h at room temperature. Nuclei were stained with DAPI. Slices were mounted with antifade mounting medium (Beyotime, Shanghai, China) and analyzed using Olympus confocal microscope (FV 1000).

2.5. Fluorescent dye transfer assay of gap junction activity

The H9c2 cells were bathed in Hanks' balanced salts solution (HBSS, Gibco, in mM): 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2PO_4, 10 D-glucose and 4.2 $NaHCO_3$. The pH and osmolarity of the bath and the pipette filling solution were adjusted to 7.4 and 295 mOsmol/L, respectively. Microelectrodes (tip diameter, ~1 μm) were pulled from capillaries and backfilled with Lucifer yellow (0.5%) dissolved in the pipette filling solution (in mM): 100 K-gluconate, 40 KCl, 5 Na_2ATP, 2.5 $MgCl_2$, 0.25 $CaCl_2$, 1 BAPTA, 0.2 cGMP, 1 glucose and 10 HEPES. Whole-cell patch-clamp configuration was established on one of the H9c2 cells within the monolayer on a cover slip, where the cells were grown to over 90% confluence. Pipette filling solution containing 0.5% Lucifer yellow was allowed to directly diffuse into the cell under whole-cell configuration and through the gap junction channels into the adjacent cells. Ten minutes after establishment of the whole cell configuration, the number of dye-coupled cells was counted using a fluorescence microscope to judge the activity of the Cx43 gap junction channels. MβCD or MβCD/Chol was used to increase or deplete the cholesterol content of the cells [13].

2.6. Statistical analysis

All data were shown as mean ± s.e.m. Data were analyzed statistically by one-way analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons. Origin7.0 (Microcal Software, Inc.) or Prism 5.0 (GraphPad Software, Inc) software was used for statistical analyses. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of cholesterol enrichment on phosphorylation, distribution and function of Cx43

First, we studied the effect of cholesterol enrichment on the function of Cx43 in cultured H9c2 cells. The function of Cx43 was evaluated by means of dye transfer ability following the intracellular microinjection of Lucifer yellow. Enrichment of cholesterol by 1 hour exposure of 50 μM cholesterol-loaded MβCD (MβCD/Chol) reduced the dye transfer ability of Cx43 (Fig. 1A). In untreated cells, the injected dye spread into 19.9 ± 1.2 ($n = 10$) cells, while the dye spread into only 5.1 ± 0.6 ($n = 10$) cells in the cholesterol enriched cells (Fig. 1 C). Immunostaining images of the cell probed with anti-Cx43 antibody revealed that the cholesterol enrichment redistributed Cx43 at the boundaries between the cells from a condensed manner for the control (e.g., the white arrow in Fig. 1B_1) to a dispersed manner for the cholesterol enriched cells (e.g., the white arrow in Fig. 1B_2). This result suggested that cholesterol enrichment caused the redistribution of Cx43 and disassembly of the Cx43 gap junction channels, resulting in the reduction of the dye transfer ability. Western blot analysis indicated that the cholesterol enrichment promoted phosphorylation of Cx43 (p-Cx43) in a dose-dependent manner (Fig. 1D): the phosphorylated Cx43 was significantly increased, while the expression level of control protein GAPDH was unchanged with increasing concentration of MβCD/Chol from 0, 2, 5, 10 to 50 μM. This result is in good agreement with a previous report that cholesterol-enriched diet results in redistribution of Cx43 gap junctions at the lateral cell membrane [4], suggesting that cholesterol enrichment could decrease dye transfer ability and increase p-Cx43 level in cultured H9c2 cells.

3.2. PKC antagonists increased dye transfer ability and reduced p-Cx43 level in cholesterol enriched cells

Next, to test whether PKC is involved in the reduction of dye transfer ability of Cx43 in the cholesterol enriched cells, we used broad spectrum PKC antagonists, STS and calphostin C, to block the effect of PKC. In the cholesterol enriched cells, the dye spread into only 5.1 ± 0.6 ($n = 10$) cells. STS (10 nM) and calphostin C (100 nM) increased the number of dye-coupled cells to 12.0 ± 1.1 ($n = 5$) and 17.4 ± 2.0 ($n = 5$), respectively (Fig. 2A and C). Immunofluorescent images showed that both STS and calphostin C redistributed Cx43 from the diffused manner (e.g., white arrows in Fig. 2B_1) to the condensed manner (e.g., white arrows in Fig. 2B_{2-3}) in the interfaces between the cholesterol enriched cells. Furthermore, western blot analysis indicated that both STS and calphostin C reduced the p-Cx43 level (Fig. 2D and E) in the cholesterol enriched cells. In contrast, STS and calphostin C alone had no significant effect on the dye-coupling ability, Cx43 distribution, Cx43 expression and p-Cx43 level in the untreated cells (Fig. 3). Thus, PKC antagonists could increase dye transfer ability, and reduce the p-Cx43 level in the cholesterol enriched cells.

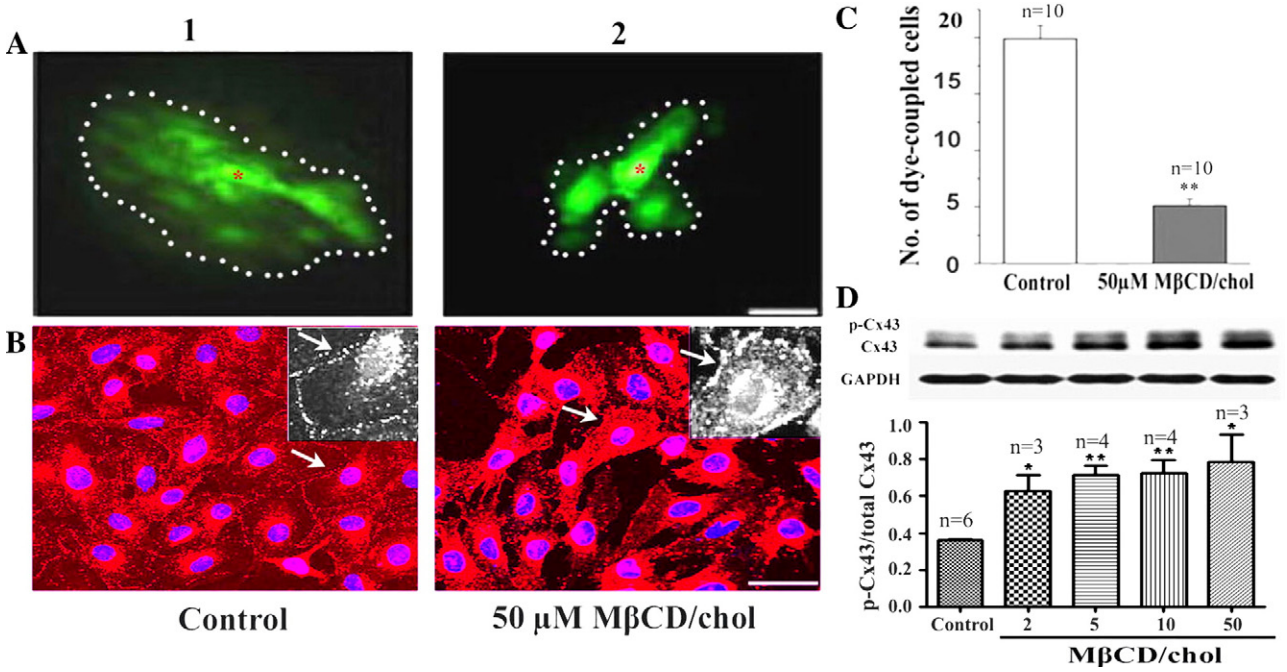

Fig. 1. Cholesterol enrichment decreased dye transfer ability and increased phosphorylated Cx43 (p-Cx43) level in cultured H9c2 cells. (A) Representative fluorescent image of dye-coupling between the cells in the absence (1) and presence (2) of 50 μM MβCD/Chol for 1 h. The H9c2 cells were grown to over 90% confluence. Dotted line: dye-coupled cells. Red asterisk: dye injected cell. (B) Representative immunofluorescent images of Cx43 (red). Nuclei: blue (DAPI). The area indicated by the white arrows is amplified as black and white images in the upright corner of the images. (C) Statistical summary for the number of dye coupled cells in the absence and presence of 50 μM MβCD/Chol. (D) Representative western blot and densitometric analyses of p-Cx43/Cx43 at control condition and in the presence of 2, 5, 10 and 50 μM MβCD/Chol. *: $P < 0.05$, **: $P < 0.01$ (vs. control).

3.3. Cholesterol depletion increased dye transfer ability and decreased p-Cx43 level in cells pre-treated with PKC agonist

To further confirm the involvement of PKC in the effect of cholesterol on Cx43, we applied PKC agonist phorbol 12-myristate 13-acetate (PMA) to the H9c2 cells. With increasing concentration of PMA from 5, 10 to 20 nM, the p-Cx43 level was significantly increased (Fig. 4D). Phosphorylation of Cx43 has been shown to be responsible for Cx43 disassembly and reduction of dye transfer ability of the cells [7,8,14–16]. Thus, when PMA (50 nM) was added, PMA reduced the number of dye-coupling cells from 19.9 ± 1.2 ($n = 10$) of the control (Fig. 1C) to 1.4 ± 0.3 ($n = 10$) in PMA treated cells (Fig. 4 C). However, reduction of the cholesterol level with MβCD could prevent the PMA induced reduction of the dye coupling between the cells (comparing Fig. 4A_1 and A_3). The number of dye-coupling cells could be increased from 1.4 ± 0.3 in PMA treated cells to 4.3 ± 0.8 ($n = 8$) in PMA + MβCD treated cells (Fig. 4C). To further confirm this result, we administered fenofibrate (Feno), a PPar-α activator, which are widely used in the treatment of hyperlipidemia and effective in lowering the cholesterol levels [17,18]. As shown in Fig. 4A2 and C, the number of dye-coupled

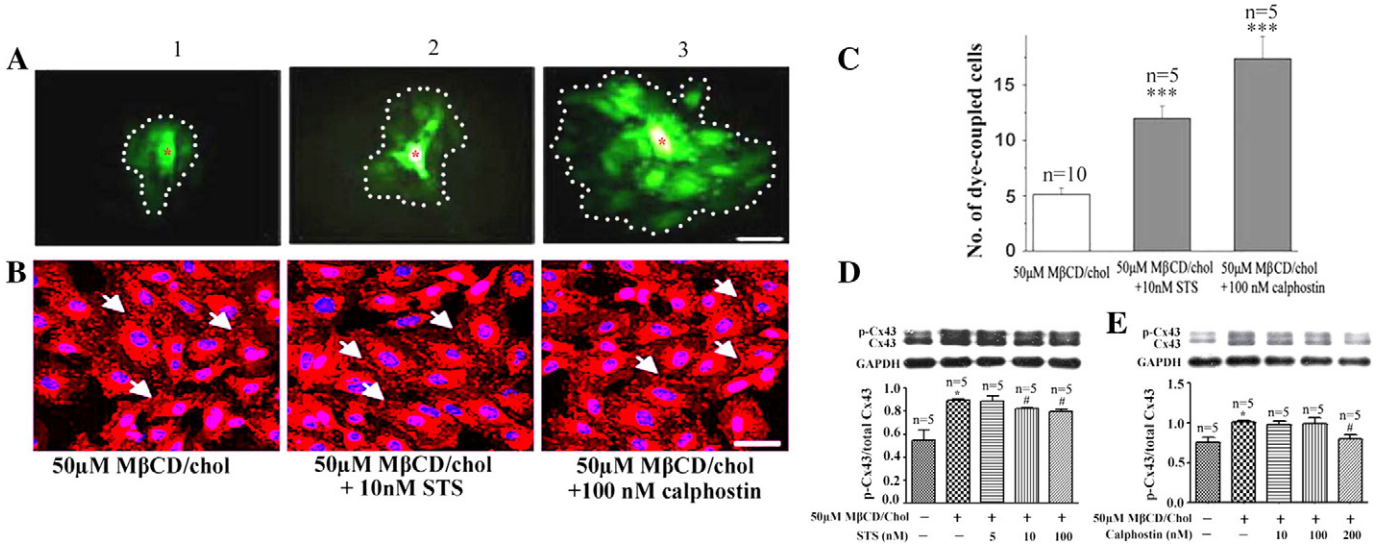

Fig. 2. PKC antagonist increased dye transfer ability and reduced p-Cx43 level in cholesterol enriched H9c2 cells. (A, B) Fluorescent image of dye-coupling between the cells (A) and immunofluorescent image of Cx43 with the white arrows indicated the changes of the Cx43 distribution (B) in the absence and presence of STS (10 nM, 30 min) or calphostin C (100 nM, 30 min). Dotted line: dye-coupled cells. Red asterisk: dye injected cell. Bar = 50 μm. (C) Statistical summary for dye coupled cells. (D, E) Western blot and densitometric analyses of p-Cx43/Cx43. Cells were treated with 50 μM MβCD/Chol for 30 min, then different concentrations of STS or calphostin C were applied for another 30 min. ***: $P < 0.001$ (vs. control group), #: $P < 0.05$ (vs. MβCD/Chol group).

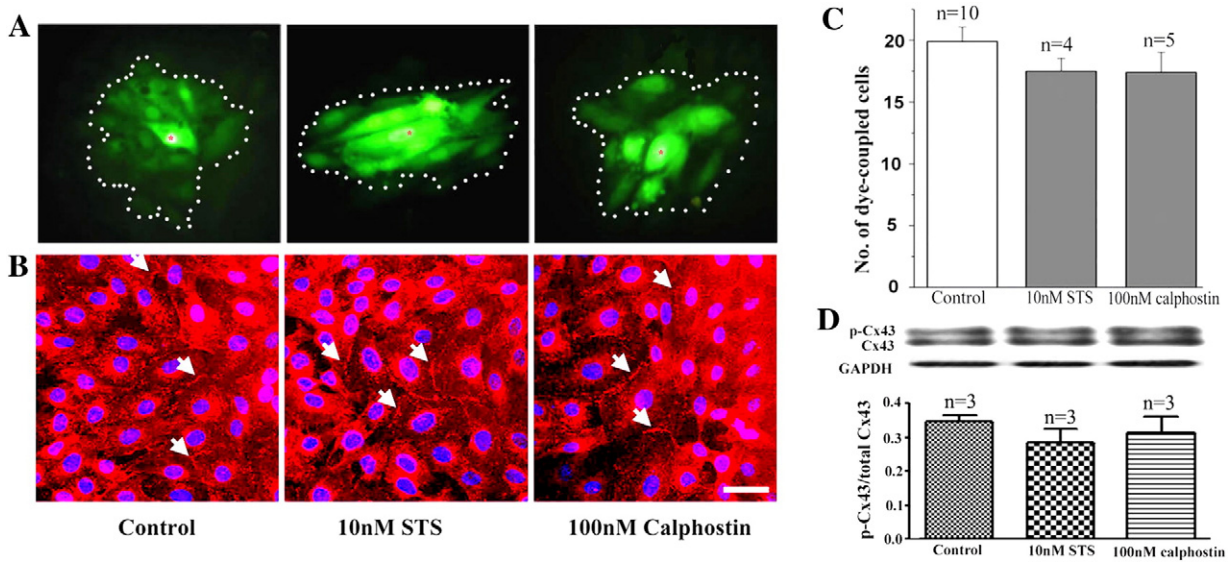

Fig. 3. Effect of PKC antagonists on dye transfer and p-Cx43 level in cultured H9c2 cells. (A, B) Fluorescent images for dye coupling between the cells (A) and immunofluorescent image of Cx43 with the white arrows indicating Cx43 distribution (B) in the absence and presence of 10 nM STS or 100 nM calphostin C. Dotted line: dye-coupled cells. Red asterisk: dye injected cell. Bar = 50 μm. (C, D) Statistical summary for dye coupled cells (C) and western blot for Cx43/p-Cx43 (D) in the absence and presence of STS and calphostin C.

cells was increased from 1.4 ± 0.3 in PMA treated cells to 3.9 ± 0.6 ($n = 8$) in PMA + Feno treated cells (Fig. 4C). This is accompanied by restoration of Cx43 distribution from a dispersed manner (e.g., white arrows in Fig. 4B_2) to a condensed manner (e.g., white arrows in Fig. 4B_3) between the boundaries of the cells. Furthermore, application of MβCD (10 mM) or Feno reduced the p-Cx43 level in the PMA pre-treated cells (Fig. 4D). In contrast, the cholesterol depletion alone (10 mM MβCD or 100 μM Feno) led to the reduction of the dye transfer ability of the Cx43 gap junction among cells (Fig. 5A and B). Therefore, cholesterol depletion could increase the dye transfer ability and decrease the p-Cx43 level in the PMA pre-treated cells.

3.4. Cholesterol enrichment upregulated the phosphorylated state of Cx43 at Ser368 through the PKC signaling pathway

It has been shown that activation of PKC causes phosphorylation of Cx43 at Ser368, which leads to disassembly of Cx43 and results in the reduction of the dye transfer ability of the cells [7,14,16]. The above results indicated that cholesterol could modulate the function of the Cx43 gap junction channel via the PKC signaling pathway. Therefore, we tested whether phosphorylation of Ser368 by PKC was responsible for the cholesterol regulation of Cx43. Fig. 6A showed that phosphorylation of Cx43 at Ser368 was significantly increased upon cholesterol enrichment. In contrast, in the presence of PKC antagonists, the cholesterol

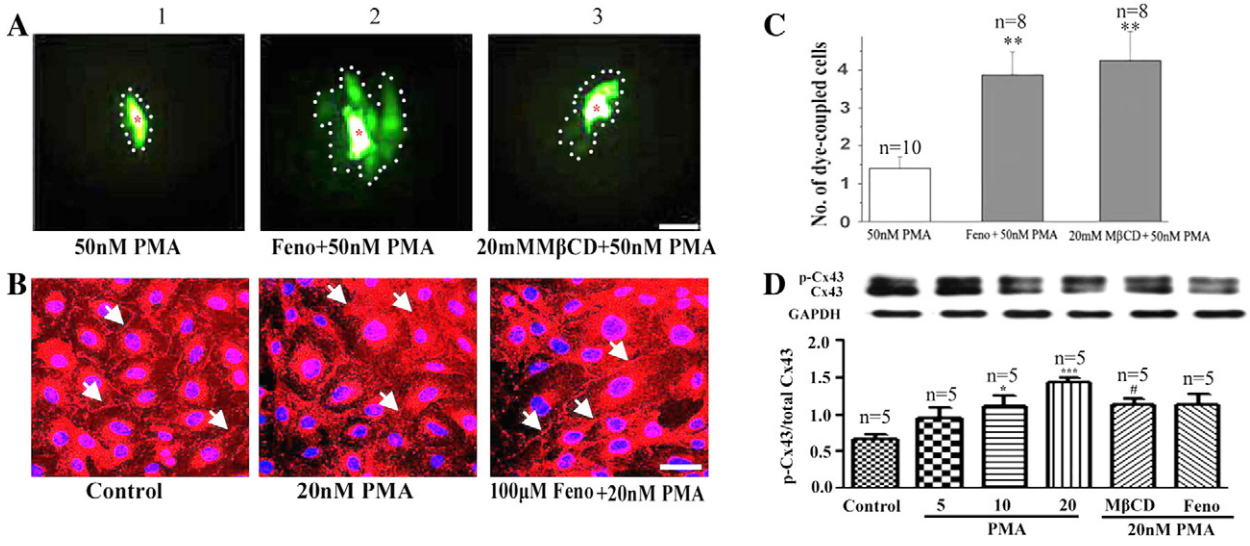

Fig. 4. Reduction of the cholesterol level increased dye transfer ability and decreased the p-Cx43 level in PKC agonist treated cells. (A, B) Fluorescent image of dye-coupling between cells (A) and immunofluorescent image of Cx43 with the white arrows indicated the changes of the Cx43 distribution (B) under the indicated conditions. Dotted line: dye-coupled cells. Red asterisk: dye injected cell. Bar = 50 μm. (C) Statistical summary for dye coupled cells. (D) Western blot and densitometric analyses of p-Cx43/Cx43. Cells were pre-treated MβCD for 30 min or Feno for 11.5 h, followed by treatment with PMA for another 30 min. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ (vs. control). #: $P < 0.05$ (vs. 20 nM PMA group).

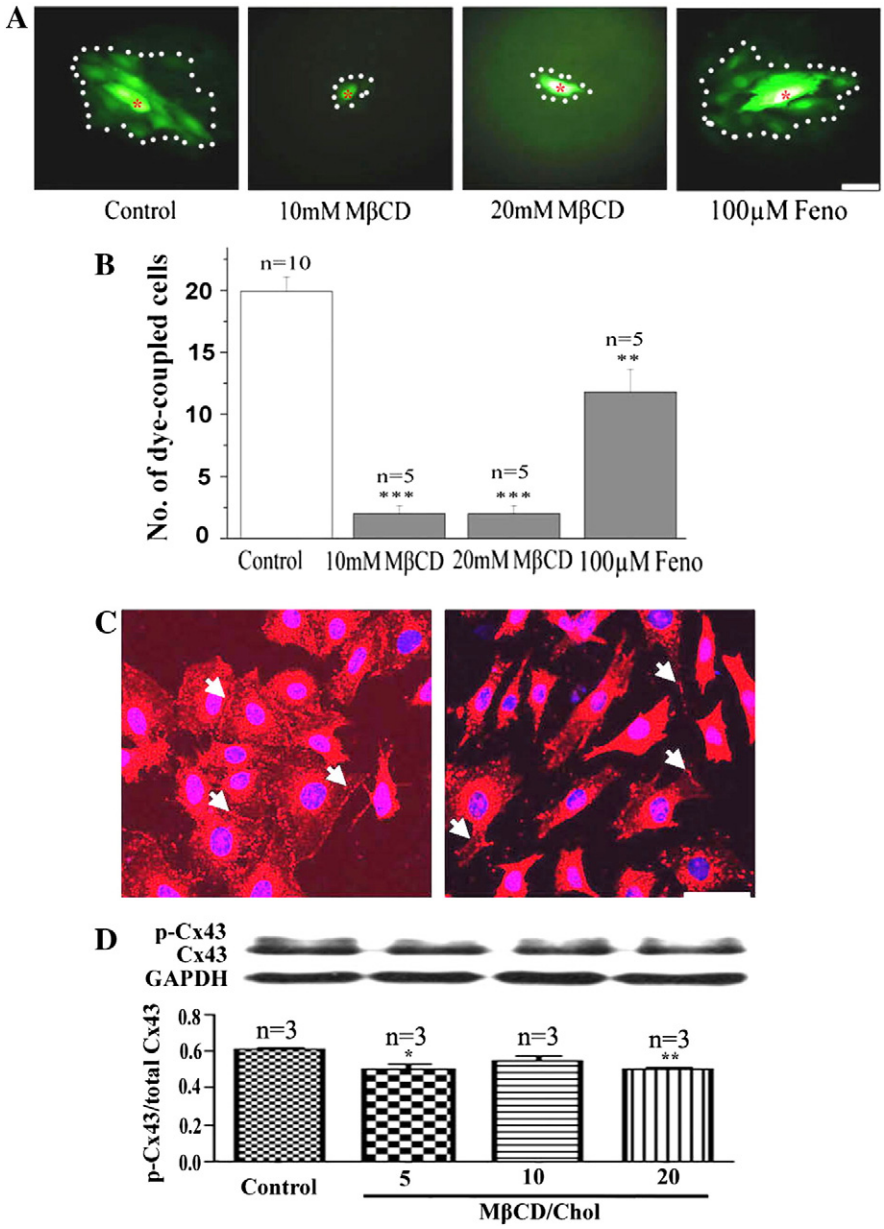

Fig. 5. Effect of cholesterol depletion on dye transfer and p-Cx43 level in H9c2 cells. (A, B) Fluorescent images (A) and statistical summary (B) for dye coupling between the H9c2 cells in the absence and presence of cholesterol lowering agents (20 mM MβCD, 1 h or 100 μM Feno, 12 h). Dotted line: dye-coupled cells. Red asterisk: dye injected cell. (C) Immunofluorescent image of Cx43 with the white arrows indicated the changes of the Cx43 distribution in the absence and presence of MβCD. (D) Western blots and densitometric analyses of p-Cx43/Cx43 in the presence of 20 mM MβCD (30 min). Bar = 50 μm.*: $P < 0.05$, **: $P < 0.01$. ***: $P < 0.001$ (vs. control).

enrichment no longer increased the Ser368 phosphorylation. On the other hand, PKC agonist PMA significantly increased the Cx43 phosphorylation at Ser368 that could be downregulated by depletion of cholesterol level with MβCD or Feno (Fig. 6B). These results demonstrated that cholesterol could modulate the function of Cx43 gap junction channel by enhancing the phosphorylation of Cx43 at Ser368 via the PKC pathway.

4. Discussion

In this work we demonstrated that cholesterol could modulate the function of the Cx43 gap junction channel by enhancing phosphorylation of Cx43 at Ser368 residue through the PKC signaling pathway, which is supported by the following pieces of evidence: 1) cholesterol enrichment increased the p-Cx43 level that could be reduced by PKC antagonists; 2) cholesterol enrichment decreased dye transfer ability that could be reversed by treatment of PKC antagonists; 3) cholesterol depletion increased dye transfer ability and decreased the p-Cx43 level in the cells pre-treated with the PKC agonist; 4) the p-Cx43 level at Ser368 upregulated by cholesterol enrichment could be downregulated by PKC antagonists. In consistence with our results, cholesterol supplementation has been shown to increase PKC activity [9] and a high level of PKC activity has been demonstrated in smooth muscle cells obtained from the aorta of cholesterol-fed rabbits [10]. Even though Cx43 is phosphorylated at multiple residues [21], PKC phosphorylates Cx43 mainly at Ser368 residue [22]. And phosphorylation of Cx43 at Ser368 is negatively correlated with gap junction assembly and reduces the cell-to-cell communication [14,15,23]. Furthermore, the HDL particle has been shown to promote phosphorylation of Cx43, which can be inhibited by pre-treatment with PKC inhibitors [8]. Interestingly, simvastatin, one of the cholesterol-lowering lipophilic statins, can enhance gap junction coupling by decreasing PKC-mediated Cx43 phosphorylation [24]. These results suggested that cholesterol could enhance the activity of PKC to phosphorylate Cx43 at Ser368, resulting in

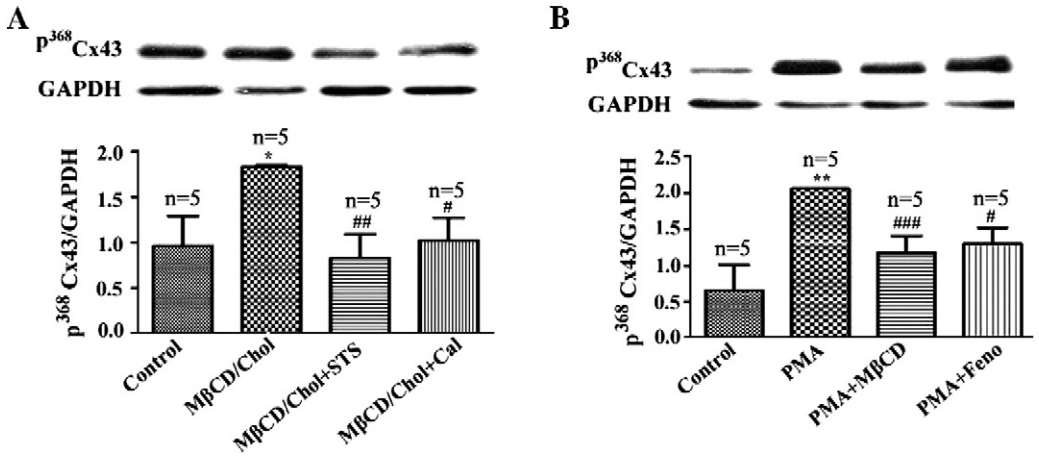

Fig. 6. Cholesterol enrichment upregulated the phosphorylated state of Cx43 at Ser368 through the PKC signaling pathway. (A) Effect of MβCD/Chol, MβCD/Chol + STS and MβCD/Chol + calphostin C on the phosphorylated state of Cx43 at Ser368. *: $P < 0.05$ (vs. control). #: $P < 0.05$, ##: $P < 0.01$ (vs. MβCD/Chol group). (B) Effect of PMA, PMA + MβCD and PMA + Feno on the phosphorylated state of Cx43 at Ser368. **: $P < 0.01$ (vs. control). #: $P < 0.05$, ###: $P < 0.001$ (vs. PMA group). Upper panel: Western blot. Lower panel: statistics summary for ratio between Ser368 phosphorylation and GAPDH.

Cx43 gap junction disassembly and reduction of the cell-to-cell communication.

Then, how does cholesterol enhance PKC activity? Cholesterol has been shown to affect membrane protein structure and function both by binding directly to specific binding sites on the protein and by changing various biophysical properties of the bilayer [25]. It is known that PKC activity is dependent on the nature of the membrane environment [26–28]. In their series studies [11,27–29], Zidovetzki et al. demonstrated that PKC activity can be increased by lipophilic compounds that can destabilize bilayers by increasing their propensity to adopt the nonbilayer lipid phase. In the phosphatidylcholine/phosphatidylserine bilayer system, amplified PKC activity was observed in the presence of both cholesterol and diacylglycerol [11]. Cholesterol has small polar headgroup and large hydrophobic body. Molecular dynamic simulation of the lipid bilayer membrane has demonstrated that to avoid the unfavorable exposure of the hydrophobic part to water, neighboring phospholipid headgroups move toward cholesterol [30]. Therefore, as suggested by ^{2}H NMR measurements, amplification of the PKC activity by cholesterol is due to the ability of cholesterol to induce propensity of the lipids to adopt nonbilayer phases, leading to destabilization of the bilayer structure [11]. As Ser368 of Cx43 is the main target residue of PKC, upregulated PKC activity could lead to an increase in the p-Cx43 level and a decrease in the cell-to-cell communication.

Cholesterol enrichment decreased dye transfer ability and increased p-Cx43 level in cultured H9c2 cells (Fig. 1). Paradoxically, cholesterol depletion (10 mM MβCD or 100 μM Feno) led to the reduced dye transfer ability of Cx43 gap junction among cells as well (Fig. 5A and B). However, western blot analysis indicated that the cholesterol depletion reduced p-Cx43 in a dose-dependent manner (Fig. 5D), which was opposite to that of the cholesterol enrichment. Furthermore, immunofluorescent images showed that the cholesterol depletion reduced the amount of Cx43 protein to distribute along the boundaries between the cells (Fig. 5C) rather than making Cx43 distribute in a dispersed manner as done by the cholesterol enrichment, suggesting different mechanisms responsible for the reduced diffusion of fluorescent dye among cells for enrichment and depletion of cholesterol. Cholesterol depletion reduced the amount of Cx43 in the interface between the cells, which might be the reason why the dye transfer ability of Cx43 was reduced, while cholesterol enrichment promoted the phosphorylation of Cx43, leading to the disassembly of Cx43 gap junction channel and resulting in reduced dye transfer ability.

Cholesterol is the principal constituent of lipid rafts. Its removal from the cell membrane would disrupt the integrity of the raft [19], thereby leading to a loss of functions mediated by these domains. Connexins that are the structural proteins of gap junctions are preferentially localized in the lipid rafts of the plasma membrane [1,16,20], which are enriched in cholesterol and sphingolipids. It has been demonstrated that membrane cholesterol plays a significant role in regulating function of gap junction channels [2,3]. Cx43 specifically targets lipid rafts by directly interacting with caveolin-1 [1]. It has been demonstrated that Cx43 is redistributed within the lipid rafts once phosphorylated by PKC at Ser368, leading to the disassembly of Cx43 out of cell junction plaques and resulting in the inhibition of dye transfer [16]. In our study, MβCD could suppress PMA activated PKC activity to phosphorylate Cx43 and restore Cx43 function. As MβCD is generally used to disrupt lipid raft [13], it is possible that MβCD takes action by disrupting the raft. On the other hand, Feno that has not been reported to disrupt lipid raft could suppress the PMA upregulated p-Cx43 level and restore the dye transfer ability of the cells, suggesting that it is cholesterol rather than raft that plays an important role in regulating Cx43 function. In agreement with this suggestion, it has been shown that cholesterol could amplify PKC activity in the phosphatidylcholine/phosphatidylserine bilayer system [11], where there is no lipid raft. Obviously, further works need to be done to address this issue. Previously, it has been demonstrated that PKC can spatially and temporally regulate gap junctional communication [14,15,23]. On the other hand, it has been shown that cholesterol plays an important role in the proper function of Cx43 [2,4]. However, it is not known whether the cholesterol level regulates the function of gap junctional communication via the PKC signaling pathway. Our study added new information by demonstrating that it is through the PKC pathway to phosphorylate Cx43 at Ser368 that cholesterol levels could modulate the function of the Cx43 gap junction channel.

Acknowledgments

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