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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.