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Connexin43 hemichannels contributes to the disassembly of cell junctions through modulation of intracellular oxidative status

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ABSTRACT

Connexin (Cx) hemichannels regulate many cellular processes with little information available regarding their mechanisms. Given that many pathological factors that activate hemichannels also disrupts the integrity of cellular junctions, we speculated a potential participation of hemichannels in the regulation of cell junctions. Here we tested this hypothesis. Exposure of renal tubular epithelial cells to Ca^{2+}-free medium led to disassembly of tight and adherens junctions, as indicated by the reduced level of ZO-1 and cadherin, disorganization of F-actin, and severe drop in transepithelial electric resistance. These changes were preceded by an activation of Cx43 hemichannels, as revealed by extracellular efflux of ATP and intracellular influx of Lucifer Yellow. Inhibition of hemichannels with chemical inhibitors or Cx43 siRNA greatly attenuated the disassembly of cell junctions. Further analysis using fetal fibroblasts derived from Cx43 wide-type ($Cx43^{+/+}$), heterozygous ($Cx43^{+/-}$) and knockout ($Cx43^{-/-}$) littermates showed that Cx43-positive cells ($Cx43^{+/+}$) exhibited more dramatic changes in cell shape, F-actin, and cadherin in response to Ca^{2+} depletion, as compared to Cx43-null cells ($Cx43^{-/-}$). Consistently, these cells had higher level of protein carbonyl modification and phosphorylation, and much stronger activation of P38 and JNK. Hemichannel opening led to extracellular loss of the major antioxidant glutathione (GSH). Supplement of cells with exogenous GSH or inhibition of oxidative sensitive kinases largely prevented the above-mentioned changes. Taken together, our study indicates that Cx43 hemichannels promote the disassembly of cell junctions through regulation of intracellular oxidative status.

1. Introduction

Maintenance of normal structure and function of tissues and organs requires complicated cell-to-cell and cell-to-matrix interactions. These interactions occur at the special contact sites called cell junctions, which include tight junctions (TJs), adherens junctions (AJs) and gap junctions (GJs) [1–4]. TJs seal the cells together and serve as a physical barrier to prevent solutes and water passing through the para-cellular space. AJs mechanically link cell to its neighboring cells or extracellular matrix, thus contributing to the maintenance of tissue integrity and cell morphology. GJs allow the intercellular exchange of small molecules, which is indispensable for the control of tissue hemostasis and coordination of multicellular functions. Disruption of cell junctions has been reported in many pathophysiological situations and has been shown to be critical involved in the initiation and development of many diseases, including infection, inflammation and tumor.

Cell junctions are composed of the transmembrane proteins and intracellular components. TJs consist of transmembrane protein occludin, claudin and the cytoplasmic scaffolding ZO proteins [1,2]. AJs are built from transmembrane protein cadherin and intracellular catenin proteins [5]. GJs are formed by transmembrane protein connexin (Cx), the c-terminal of which interacts with ZO-1 and other cytoskeleton proteins [6,7]. Besides their distinct roles, these junctions also function as a signaling platform, regulating cellular processes, such as differentiation, growth and migration, through interactions with actin cytoskeletal elements, kinases and phosphatases [2,8].

The assembly and disassembly of cell junctions are tightly regulated in an orchestrated way. The mechanisms involved are

Abbreviations: Cx, connexin; Cx43, connexin43; TJs, tight junctions; AJs, adherens junctions; GJs, gap junctions; siRNA, small interfering RNA; $Cx43^{+/+}$, Cx43 wide-type mouse; $Cx43^{+/-}$, Cx43 heterozygous mouse; $Cx43^{-/-}$, Cx43 knockout mouse; JNK, c-Jun N-terminal kinase; GSH, glutathione; FBS, fetal bovine serum; LY, lucifer yellow; SB203580, 4-(4-fluorophenyl)−2-(4-methylsulfinylphenyl)−5-(4-pyridyl) 1H-imidazole; SP600125, 2H-Dibenzo[cd,g]indazol-6-one; NAC, N-acetyl-cysteine; NRK, Normal rat kidney; SDS, sodium dodecyl sulphate; DNPH, 2,4-dinitrophenylhydrazine; OD, optical density; TER, transepithelial electrical resistance

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complicated and multiple [1–4]. Numerous studies indicate an existence of the coordinated inter-regulation among junctional proteins. For examples, AJs have been documented to be requisite for formation of TJs and GJs. Antibody against adherens junction proteins prevented the assembly of TJs [9,10], and GJs [11,12]. On the other hand, deletion of TJ protein ZO-1 disrupted cadherin-dependent AJs [5] and interfered with the formation of functional GJs [13]. It is conceivable that GJs might also regulate TJs and AJs. However, the evidence supporting this notion is still limited.

GJs are clusters of transmembrane channels that permit the direct exchange of ions, secondary messengers, and small signaling molecules among adjacent cells. Each GJ channel is composed of two hemichannels that reside in the plasma membrane of two closely apposed cells. Each hemichannel is made of six connexins (Cxs). Currently, more than 20 different isoforms of Cx have been identified. Among them, Cx43 is the most extensively investigated one because of its predominant and ubiquitous expression in almost all the cell types. Cx channels regulate many aspects of cell functions. Most of the effects are mediated by gap junctional intercellular communication (GJIC) between adjacent cells. Under certain conditions, the non-junctional hemichannels are activated, which also affects cell phenotypes through permitting exchange of molecules between the inside and outside of the cell [3,14–19].

Several considerations promoted us to speculate that GJs, especially Cx hemichannels, might contribute to the disassembly of cell junctions. First, cell junctions are in close proximity to each other in polarized cells. They all linked to scaffolding and cytoskeletal proteins [20]. The change of one type of junction may affect the others. Second, modulation of GJs or its forming proteins activate several important signaling molecules, including those critically involved in the control of cell junctions, such as src and MAP kinases [8,21–23]. Third, the stimuli that activate hemichannels, such as depolarization of the membrane potential, hypoxia, metabolic inhibition, inflammation, change of intracellular and extracellular Ca^{2+}, as well as cellular redox status [3,24–28], are also report to induce the disassembly of cell junctions. Fourth, several lines of evidences indicate that GJs regulate oxidative stress [15,19,29–31], whereas oxidative stress is one of the major mechanisms behind the dysfunction of cell junctions under many different pathological situations [32–35]. It is, therefore, highly probable that Cx channels regulate cell junctions. This study was designated to address this speculation.

Here we present our finding that Cx43 hemichannels regulate cell junctions, cytoskeleton organization, and cell shape through modulation of intracellular oxidative status.

2. Materials and methods

2.1. Reagents

GSH-Glo™ assay kit was purchased from Promega (Madison, WI). Heptanol, lindane, suramin, Lucifer Yellow (LY), SB203580, SP600125, glutathione (GSH), N-acetyl-cysteine (NAC), and all other chemicals were obtained from Sigma (Tokyo, Japan). Antibodies against ZO-1, p38, JNK and β-actin were obtained from Cell Signaling (Beverly, MA).

2.2. Cells

Normal rat tubular epithelial cell line NRK-E52 was purchased from ATCC. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS).

Mouse embryonic fibroblasts were derived from the fetal offspring of mating pairs of heterozygous Cx43 knock-out mice (B6, 129-*Gjal*-tm 1 $Kdr^{-/-}$; The Jackson Laboratory, Bar Harbor, ME) using a method described previously [15]. Briefly, both mouse forelimbs were taken from fetuses at day 18 of gestation, minced, and digested in DMEM/F-12 containing 0.1% collagenase for 30 min. Freed cells were collected and cultured in DMEM/F-12 medium containing 15% FBS. Cells at passages 5–15 were used for this study. Genotypes of individual mice were determined by PCR. The difference in expression of Cx43 and gap junctional intercellular communication among $Cx43^{+/+}$, $Cx43^{+/-}$ and $Cx43^{-/-}$ cells has been characterized and described in our previous reports [15,36]. The culture of mouse fibroblasts was performed with the approval of the Animal Care Committee of the University of Yamanashi.

2.3. Exposure of cells to normal and Ca2+-free condition

For comparison of cell responses under normal and Ca^{2+}-free conditions, cells were exposed to Ca^{2+}-free DMEM (Gibco-BRL, catalogue number 21,068) with or without supplementation of 1.8 mM Ca^{2+}.

2.4. Immunofluorescent staining of ZO-1 and F-actin

Cells cultured in 16-well chamber slide were stimulated with various stimulants as described in the results. Cells were fixed with 3% formaldehyde and permeabilized with 1% Triton X-100. For staining of ZO-1, cells were incubated overnight with anti-ZO-1 antibody. After rinsing with PBS, cells were incubated with the appropriate secondary antibody for 2 h. For staining of F-actin, cells were exposed to Texas Red-X phalloidin for 1 h. After washing, the slides were covered with Tris-buffered moviol and visualized using an Olympus inverted fluorescence microscope and the fluorescent images were captured using a CCD-camera attached to the microscope.

2.5. Western blot analysis

Total cellular protein was extracted by suspending the cells in sodium dodecyl sulphate (SDS) lysis buffer together with freshly added proteinase inhibitor cocktail (Nacalai tesque, Kyoto, Japan). Lysates were incubated on ice for 30 min with intermittent mixing and then centrifuged at 12,000 rpm for 10 min at 4 °C in an Eppendorf centrifuge. The supernatant was recovered and the protein concentration was determined using the Micro BCA Protein Assay Kit (The Thermo Scientific Pierce, Rockford, IL). Western blot was performed using the enhanced chemiluminescence system. Briefly, extracted cellular proteins were separated by 10% SDS polyacrylamide gels and electrotransferred onto polyvinylidine difluoride membranes. After blocking with 3% bovine serum albumin in PBS, the membranes were incubated with primary antibody for 1.5 h at room temperature or at 4 °C overnight. After washing, the membranes were probed with horseradishperoxidase-conjugated anti-rabbit or -mouse IgG antibody (Cell Signaling, Beverly, MA), and the bands were visualized by using the enhanced chemiluminescence system (Nacalai Tesque, Kyoto, Japan). The chemiluminescent signal was captured with a Fujifilm luminescent image LAS-1000 analyzer (Fujifilm, Tokyo, Japan) and quantified with the Image J software (http://rsb.info.nih.gov/ij). To confirm equal loading of proteins, the membranes were probed for β-actin.

2.6. Measurement of transepithelial electrical resistance (TER)

The NRK-E52 cells were cultured on 0.33 cm^2 cell culture inserts with 0.4 μm pore size (Millipore) to form monolayers. TER was measured with a Millicell electrical resistance system

(Millipore, Bedford, MA) using the protocol provided by the manufactory. TER was calculated from the measured voltage and normalized by the area of the monolayer. The background TER of blank Transwell filters was subtracted from the TER of cell monolayers.

2.7. Treatment of cells with siRNA

NRK-E52 cells were transiently transfected with siRNA specifically targeting Cx43 (Mm-Gja1–2 HP siRNA; Qiagen, Tokyo, Japan) or a negative control siRNA (AllStars Negative Control siRNA) at a final concentration of 20 nM using Hyperfect transfection reagent.

2.8. Evaluation of cell morphology using calcein-AM

Calcein-AM was used to facilitate the evaluation of cell morphology. Briefly, cells were exposed to Ca^{2+}-free medium in the presence or absence of the related agents for the indicated time intervals. Afterwards, cells were loaded with 100 nM calcein-AM for 1 h at 37 °C in culture medium. After washing once with the culture medium, the fluorescent image was visualized and captured using an Olympus inverted fluorescence microscope equipped with a standard green fluorescence cube.

2.9. Assessment of protein oxidation

The oxidative modification of proteins was evaluated by OxyBlot Protein Oxidation Detection Kit (EMD Millipore, Billerica, MA) following the manufacturer's manual, as described previously [29]. Briefly, the protein lysate was prepared by suspending the prewashed cells in SDS lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol) together with freshly added proteinase inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and 50 mM DTT. Five microliter of protein sample in Eppendorf tubes was denatured through mixing with same amount of 12% SDS. Afterwards, the samples were derivatized by adding 10 μl of 1 × DNPH (2,4-dinitrophenylhydrazine) solution to each tube. After incubation at room temperature for 15 min, 7.5 μl of neutralization solution was added and the samples were subjected to Western blot analysis.

2.10. Dye uptake assay

The presence of functional hemichannels was evaluated by the cellular uptake of LY, as described previously [15,19]. Briefly, cells were exposed to culture medium containing 0.1% LY for 15 min. Afterward, cells were rinsed and fixed with 3% paraformaldehyde. Immunofluorescence images were captured using a CCD camera attached to an Olympus inverted fluorescence microscope.

2.11. ATP measurement

ATP was measured using a luciferin/luciferase bioluminescence assay kit (Molecular Probes, Eugene, OR). The intensity of chemiluminescent signal was determined by a luminometer (Gene Light 55; Microtech Nition, Chiba, Japan) as described previously [15,19].

2.12. GSH measurement

Cells at confluent culture in 96-well culture plates were exposed to Ca^{2+}-free medium for 30 min. The supernatants were collected for measurement of extracellular GSH level using a GSH-Glo GSH Assay kit from Promega according to the manufacturer's instruction [15]. In brief, culture supernatants were mixed with equal volume of pre-prepared GSH-Glo reagent and placed at room temperature for 30 min. Thereafter, detection reagent containing luciferin was added and incubated for 15 min. Luminescence was read using a luminescence plate reader.

2.13. Statistical analysis

Values are expressed as mean ± SE. Comparison of two populations was made using Student's *t*-test. For multiple comparisons with a single control, one-way analysis of variance (ANOVA) followed by Dunnett's test was employed. Both analyses were carried out using SigmaStat statistical software (Jandel Scientific, CA, USA). $P < 0.05$ was considered to be a statistically significant difference.

3. Results

3.1. Cx43 hemichannels contribute to Ca^{2+} depletion-elicited disassembly of cell junctions

To determine the role of Cx channels in the disassembly of cell junctions, we first established the model of cell junction disassembly by removal of extracellular Ca^{2+}. Exposure of renal tubular epithelial cell NRK-E52 to Ca^{2+}-free medium led to a time-dependent disassembly of cell junctions, as revealed by the reduction or disappearance of ZO-1 at cell-to-cell contact region by IF staining (Fig. 1A), and the reduced level of pan-cadherin (Fig. 1B). Analysis of epithelia barrier function through measurement of TER revealed that removal of extracellular Ca^{2+} led to a time-dependent reduction in TER (Fig. 1C).

We have previously documented that Ca^{2+} depletion activates Cx hemichannels [19,37]. To determine the potential role of hemichannels in the regulation of cell junctions, we treated cells with hemichannel inhibitors. As shown in Fig. 2A, the disappearance of ZO-1 between cell contact regions was largely prevented by heptanol and lindane. These inhibitors also prevented the reduction in Pan-cadherin and TER caused by Ca^{2+} depletion (Fig. 2B and C).

In further support of a role of Cx channels, we treated cells with siRNA against Cx43, the functional Cx expressed in NRK-E52 cells [38]. Downregulation of Cx43 with siRNA delayed the disappearance of pan- and E-cadherin, and the dysfunction of epithelial barriers (Fig. 2D and E).

Cx43 siRNA and chemical inhibitor lindane and heptanol disrupt both intercellular gap junction channels and hemichannels [15,19,29,31,37,39]. To determine the role of hemichannels, we used suramin, a general P_2X and P_2Y inhibitor that blocks Cx hemichannels without great influence on intercellular gap junctional channels [37]. Fig. 2F shows that suramin also greatly prevented the reduction of Pan-cadherin initiated by the removal of calcium. These results indicate that Cx43 hemichannels contribute to Ca^{2+} depletion-triggered dysfunction of epithelial barriers.

To further establish the role and to explore the molecular mechanisms involved in the effect of Cx channels, we cultured fetal forearm fibroblasts from Cx43 wild-type ($Cx43^{+/+}$), heterozygous ($Cx43^{+/-}$) and knockout ($Cx43^{-/-}$) littermates and compared their responses to Ca^{2+} depletion. Of note, we have reported that these cells expressed different levels of Cx43 and displayed different capacity in GJIC and hemichannel activity [15,36]. Incubation of fibroblasts in Ca^{2+}-free medium resulted in cell shape change, as evidenced by the appearance of round and loosely attached cells (Fig. 3A). Examination of cell cytoskeleton through phalloidin staining of F-actin revealed that F-actin was aggregated and disorganized in $Cx43^{+/+}$ cells (Fig. 3B). Western blot analysis of pan-cadherin showed that $Cx43^{+/+}$ cells exhibited the severest loss of pan-cadherin (Fig. 3C). These results indicate that the

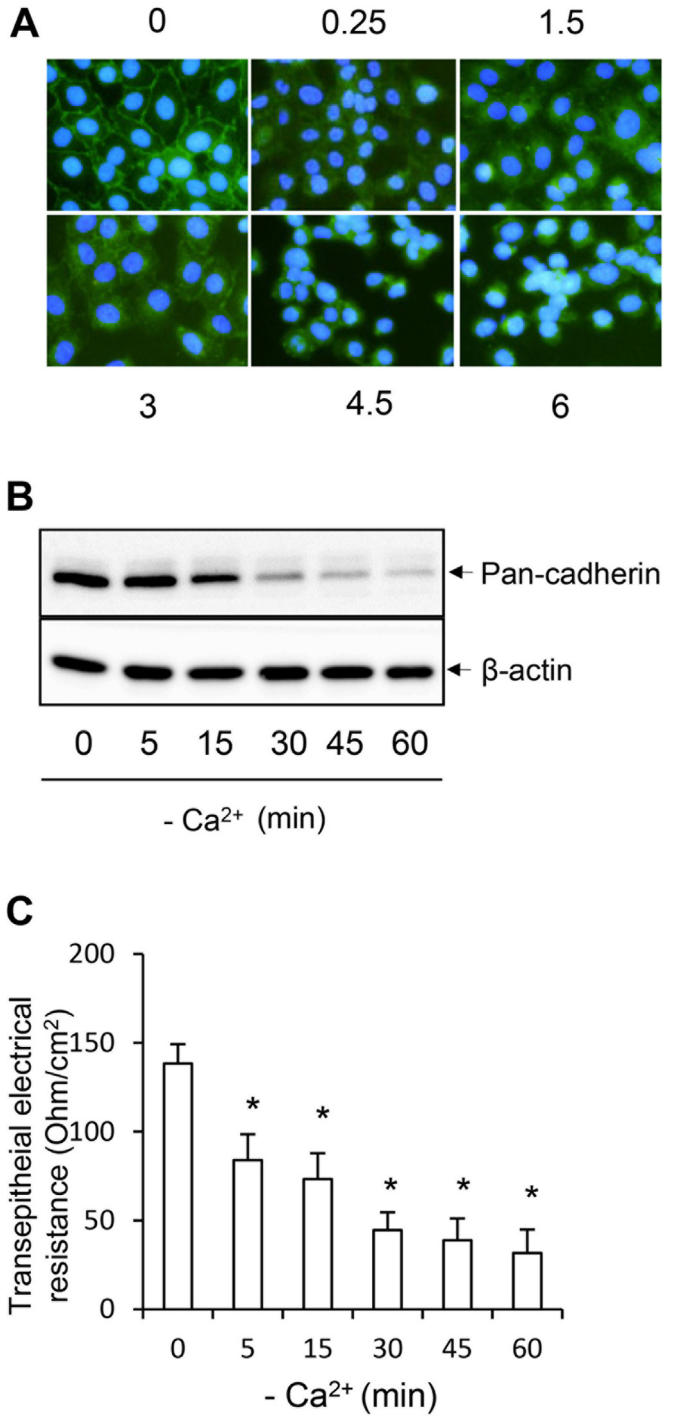

Fig. 1. Ca^{2+} depletion causes disruption of epithelia barrier. Influence of Ca^{2+} deprivation on junctional protein expression (A, B) and function (C). NRK-E52 cells were exposure to Ca^{2+}-free medium for the indicated time and subjected to immunofluorescence for ZO-1 (A), Western blot analysis for pan-cadherin (B) and TER measurement (C). β-actin in B was used as a loading control. Data shown in C are mean ± SE (n=5;* $P<0.01$ vs. control).

disassembly of cell adherens junctions is severer in Cx43-positive fibroblasts ($Cx43^{+/+}$, $Cx43^{+/-}$) than that in $Cx43^{-/-}$ fibroblasts.

In further support of a role of Cx43 hemichannels, treatment of Cx43-positive fibroblasts with Cx channel inhibitor, heptanol and lindane, largely prevented Ca^{2+} depletion-elicited changes in cell morphology and pan-cadherin (Fig. 3D and E). These observations suggest that Cx43 hemichannels play a crucial role in the disassembly of cell junctions.

3.2. Cx43 hemichannels control Ca^{2+} depletion-triggered protein tyrosine phosphorylation and carbonyl modification

Given that protein tyrosine phosphorylation plays a key role in the control of cell junction integrity, cytoskeleton organization and cell shape [40–42], we, therefore, examined the influence of Cx43 channels on tyrosine phosphorylation. Removing extracellular Ca^{2+} in $Cx43^{+/+}$ fibroblasts caused tyrosine phosphorylation of several proteins at the molecular weight around 75, 130 and 180 kDa. Intriguingly, the intensity of the peak phosphorylation appeared to be closely correlated with the amount of Cx43, being the strongest in $Cx43^{+/+}$ cells and weakest in $Cx43^{-/-}$ cells (Fig. 4A). In support of an involvement of Cx43 channels in the regulation of protein phosphorylation, inhibition of Cx43 channels with heptanol or lindane caused a reduction in tyrosine phosphorylation (Fig. 4B).

Because oxidative stress is one of the major mechanisms behind the disruption of cell junctions [22,43,44], we therefore examined level of protein carbonyls, an important and immediate biomarker of oxidative stress. As shown in Fig. 4C, exposure to fibroblasts to Ca^{2+}-free medium led to an obvious increase in protein carbonyl modification, which was more dramatic in $Cx43^{+/+}$ fibroblasts than $Cx43^{-/-}$ cells. Similar to the situation of protein phosphorylation, Cx channel inhibitor heptanol and lindane also blunted protein carbonyl modification (Fig. 4D). These results thus indicate an existence of Cx43 channel-mediated regulation of protein tyrosine phosphorylation and carbonyl modification.

3.3. P38 and JNK participate in Cx43-mediated disassembly of TJs/AJs

To investigate the signal mechanisms involved in Cx43 channel-mediated regulation of cell junctions, we focused on p38 and JNK, two oxidative sensitive kinases known to be pivotally involved in the regulation of a wide range of cell behaviors [32,45,46]. As shown in Fig. 5A, removal of extracellular Ca^{2+} led to an increased phosphorylation of p38 and JNK, which was far more pronounced in $Cx43^{+/+}$ fibroblasts than that in $Cx43^{-/-}$ cells. In support of a role of Cx43 channels in regulation of the kinases, inhibition of the channel activity in $Cx43^{+/+}$ cells with heptanol or lindane abolished the activation of P38 and JNK (Fig. 5B). These results indicate Cx43 participates in the control of Ca^{2+} depletion-elicited activation of P38 and JNK.

Several previous studies have shown that MAPK plays an important in regulation of cell junctions [32,45–47]. To determine their roles in our experimental settings, we inhibited p38 and JNK with SB203580 and SP600125, respectively. As expected, these chemicals pronouncedly inhibited Ca^{2+} deprivation-induced disassembly of cell junctions, as revealed by the better cell shape, F-actin arrangement, and protein level of pan-cadherin (Fig. 5C-E). These observations indicate that Ca^{2+} deprivation-triggered disassembly of cell junctions is through Cx43-dependent activation of p38 and JNK.

3.4. Ca^{2+} depletion activates Cx43 hemichannels and causes efflux of GSH

To investigate the mechanism in Cx43-mediated disassembly of TJs/AJs, we focused on Cx43 hemichannels, which was reported to be activated under low Ca^{2+} condition [19,37]. LY uptake assay showed that there was an influx of LY into Cx43-positive $Cx43^{+/+}$ and $Cx43^{+/-}$ fibroblasts (Fig. 6A) subsequent to the removal of extracellular Ca^{2+}. This influx was largely prevented by hemichannel inhibitor heptanol and lindane. It was also suppressed by suramin, a recently reported hemichannel inhibitor that suppresses hemichnnels with no effects on intercellular GJs (Fig. 6B) [37]. Consistently, Cx43-bearing cells released significantly more

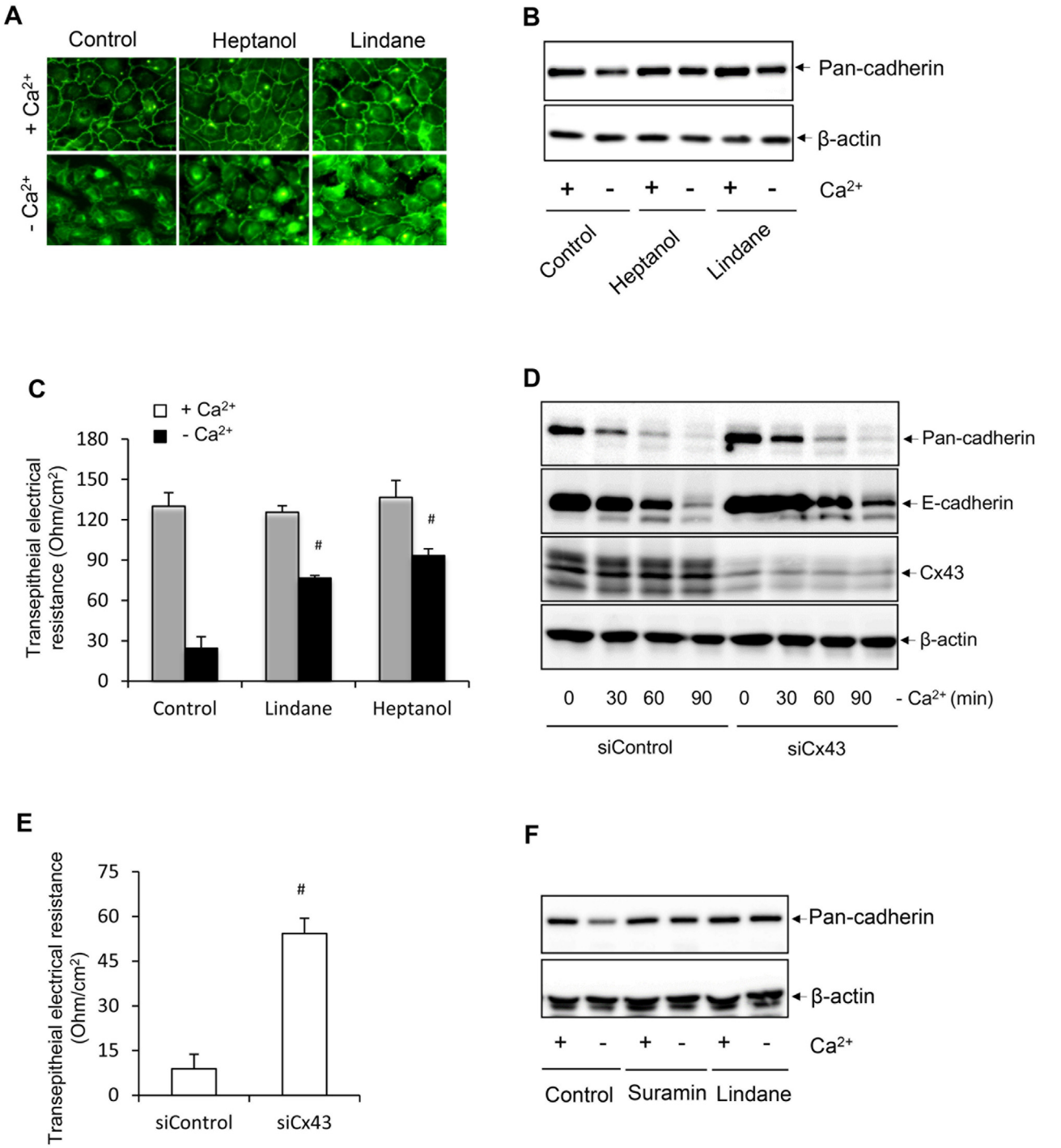

Fig. 2. Cx43 hemichannels contribute to Ca^{2+} depletion-elicited disruption of epithelia barrier. (A–C) Effect of Cx43 inhibitors on junctional protein expression and function. NRK-E52 cells were pre-treated with 3 mM heptanol and 100 μM lindane for 20 min before exposed to Ca^{2+}-free medium for an additional 1 h (A) or 30 min (B, C). Cells were subjected to immunofluorescence for ZO-1 (A), Western blot analysis for pan-cadherin (B) and measurement for TER (C). Data shown in C are mean ± SE (n=3; # $P<0.05$ vs. control). (D, E) Downregulation of Cx43 with siRNA on junctional protein expression and function. Cells were transfected with control siRNA (siControl) or siRNA against Cx43 for 48 h before exposing to Ca^{2+}-free medium for the indicated time (D) or 30 min (E). Cellular lysates were subjected to western blot analysis for cadherin or Cx43 (D). Epithelial barrier function was analyzed through measurement of TER (E). Data are mean ± SE (n=4; # $P<0.05$ vs. siControl). (F) Effect of suramin on Pan-cadherin expression. NRK-E52 cells were pre-treated with 200 μM suramin or 100 μM lindane for 20 min before exposed to Ca^{2+}-free medium for an additional 30 min. Cellular lysates were subjected to Western blot analysis for pan-cadherin.

ATP than Cx43-null cells ($Cx43^{-/-}$) under Ca^{2+}-free condition, which was preventable by hemichannel inhibitors (Fig. 6C and D). These observations indicate that Ca^{2+} depletion activates Cx43 hemichannels.

Several previous studies showed that hemichannel opening led to extracellular loss of the major antioxidant GSH [15,18]. We speculated that the loss of GSH via hemichannels might weaken cell defense against oxidative stress, thus exaggerating the disruption of cell junctions. To test this hypothesis, we first confirmed the loss of GSH through hemichannels. Fig. 6E shows that Ca^{2+} depletion caused extracellular efflux of GSH, the level of which was significantly higher in $Cx43^{+/+}$ fibroblasts than $Cx43^{-/-}$

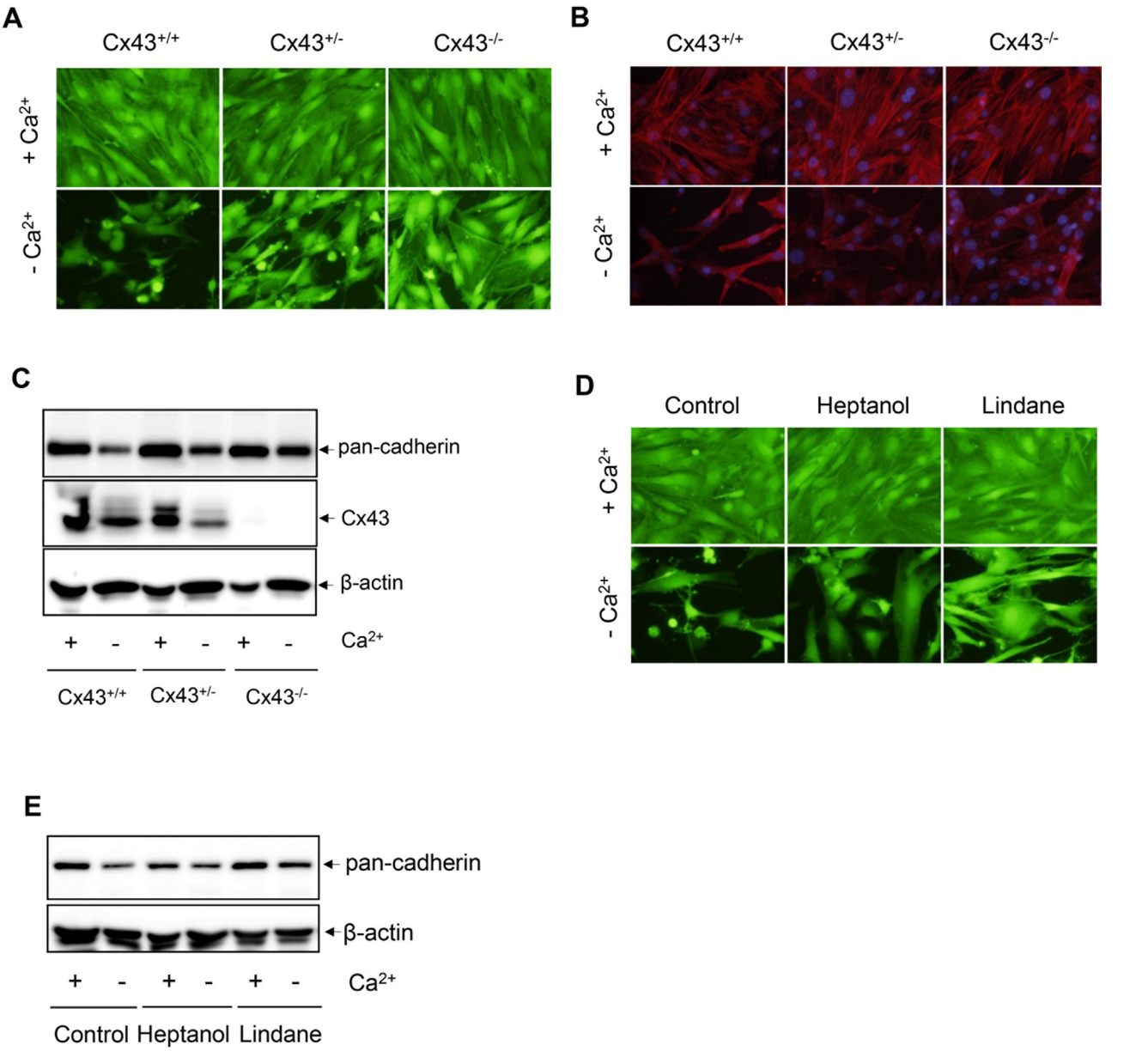

Fig. 3. Cx43 contributes to Ca^{2+} depletion-induced cell shape change and disassembly of AJs in fibroblasts. (A-C) Different responses of $Cx43^{+/+}$, $Cx43^{+/-}$ and $Cx43^{-/-}$ fibroblasts to Ca^{2+} depletion-elicited changes in cell shape, cytoskeleton organization and pan-cadherin level. (A) Fibroblasts were exposed to Ca^{2+}-free medium for 24 h. Cells were then loaded with 100 nM calcein for 1 h and cell morphology was photographed. (B) Fibroblasts were exposed to the Ca^{2+}-free medium for 10 h and subjected to phalloidin staining for F-actin (red, B). (C) Fibroblasts were exposed to Ca^{2+}-free medium for 3 h. The cellular proteins were immunoblotted for pan-cadherin, Cx43 and β-actin (C). (D-E) Inhibition of Cx43 hemichannels on cell shape change and pan-cadherin level in $Cx43^{+/+}$ fibroblasts. (D) $Cx43^{+/+}$ fibroblasts were exposed to Ca^{2+}-free medium for 24 h in the presence or absence of 3 mM heptanol or 100 μM Lindane. Cells were then loaded with 100 nM calcein for 1 h and cell morphology was photographed. (E) $Cx43^{+/+}$ fibroblasts were exposure to Ca^{2+}-free medium for 3 h and cellular lysates were subjected to Western blot analysis for pan-cadherin (E).

cells. Inhibition of hemichannels largely prevented extracellular release of GSH (Fig. 6F).

Then we proceeded to determine the role of GSH by supplement of cells with GSH or its precursor N-acetylcysteine (NAC). Fig. 7 show that GSH and NAC significantly attenuated Ca^{2+} depletion-induced alteration in cell morphology and F-actin rearrangement (Fig. 7A and B), suppressed the protein carbonyl modification, tyrosine phosphorylation and activation of oxidative sensitive kinase P38 and JNK (Fig. 7, C-E), and prevented the reduction in pan-cadherin level and FAK phosphorylation (Fig. 7F). These observations indicate that Cx43 hemichannel-mediated loss of GSH contributes to the oxidative stress and disassembly of cell junctions.

4. Discussion

In this study, we demonstrated that Cx43 hemichannels mediated Ca^{2+} deprivation-elicited disassembly of cell junctions. Furthermore, we provided evidence that this effect of hemichannels was through regulation of cellular oxidative status. Our study thus provides novel mechanistic insight into the regulation of cell junctions under pathological situations and suggests that strategies against Cx channels could be developed to limit the disruption of cell barrier.

Ca^{2+} is indispensable for maintenance of normal structure and function of cell junctions. Removal of extracellular Ca^{2+} disrupts cell junctions. It is, in fact, a simple and extensively used model for investigating the mechanisms implicated in the disassembly of cell

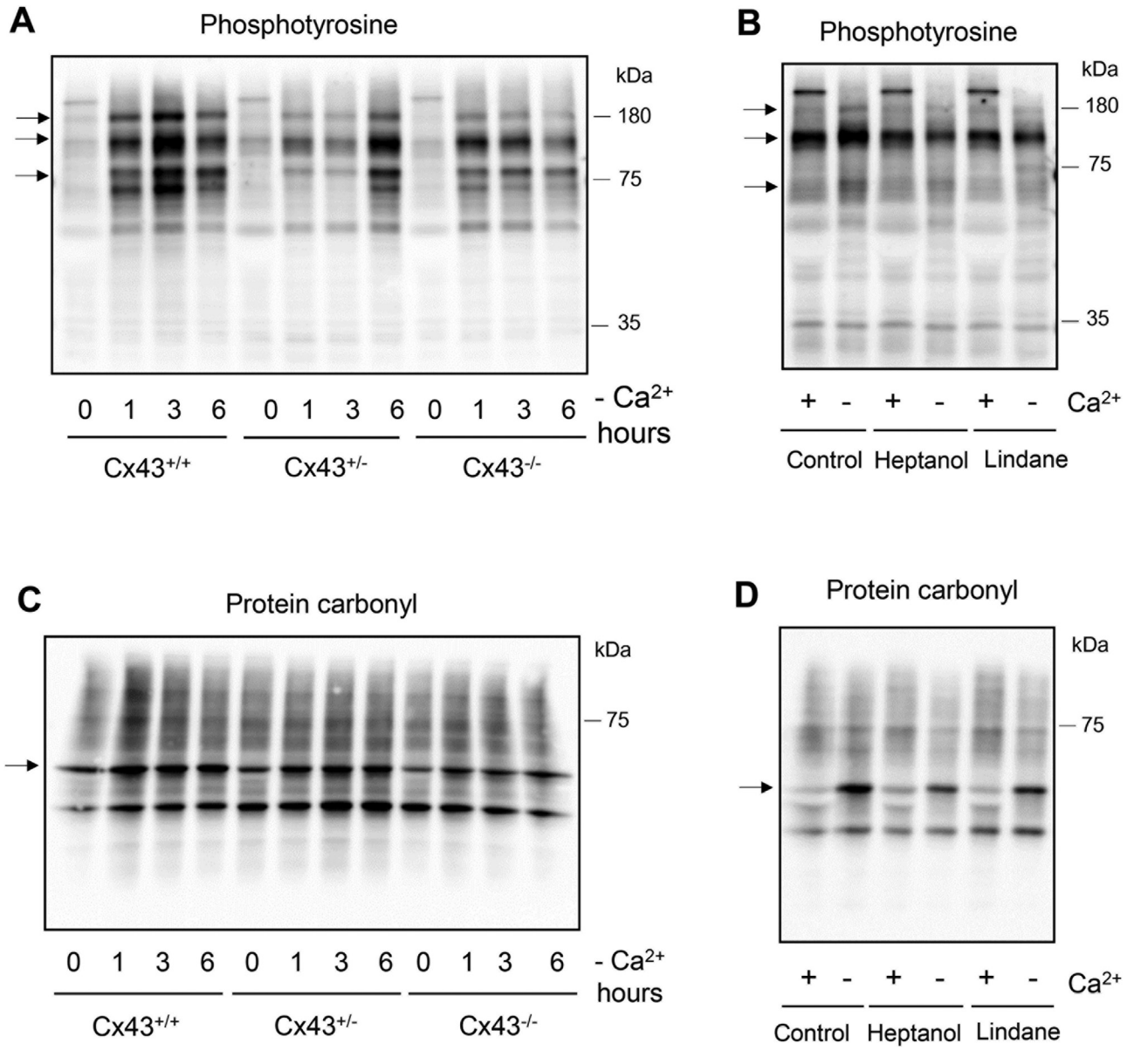

Fig. 4. Cx43 participates in Ca^{2+} depletion-induced modification of tyrosine phosphorylation and protein carbonyl. (A, C) Different responses of $Cx43^{+/+}$, $Cx43^{+/-}$ and $Cx43^{-/-}$ fibroblasts to Ca^{2+} depletion-induced tyrosine phosphorylation and protein carbonyl. Cells were exposed to Ca^{2+}-free medium for the indicated time intervals. Cellular lysates were subjected to Western blot analysis for phosphorylated tyrosine (phosphotyrosine) (A) and protein carbonyl (C). (B, D) Effects of hemichannel inhibitors on Ca^{2+} depletion-induced changes in tyrosine phosphorylation and protein carbonyl modification in $Cx43^{+/+}$ fibroblasts. Cells were exposed to Ca^{2+}-free medium for 3 h in the presence or absence of 3 mM Heptanol or 100 μM Lindane. Cellular protein was immunoblotted for phosphorylated tyrosine (B) and protein carbonyl (D).

junctions [48]. Lowering extracellular Ca^{2+} also activates hemichannels. Structural and functional studies revealed that Ca^{2+} depletion resulted in an enhanced diameter of outer hemichannel pore [49] and an increased exchange of small molecules between the intra- and extracellular environments [19,37]. The concomitant induction of hemichannel opening and cell junction disassembly by Ca^{2+} depletion made it an ideal model to correlate their relationship. Taking advantages of this model, we observed that hemichannel opening occurred earlier than the disassembly of cell junctions and that blockade of hemichannels with chemical inhibitors or downregulation of Cx43 with siRNA attenuated the disruption of cell junctions. These observations thus indicate that Cx channel activity contributed to the disassembly of cell junctions.

In this study, we have used heptanol and lindane to block Cx channel activities. These inhibitors have different chemical structure and inhibit the channel activity through different mechanisms. Heptanol blocks connexin channel formation through decreasing the open probability of the channels [50], whereas lindane works by regulating multiple intracellular signaling molecules [19,51]. Although these inhibitors also have other nonspecific actions, the observed effects were most likely the result of their common inhibitory actions on Cx channels. In line with this notion, downregulation of Cx43 with siRNA achieved the similar effects. Of note, lindane, heptanol and Cx43 siRNA also interfere with GJ channels [15,19,29,31,37,39]. In this study, it appeared that hemichannels played a major role, because suramin, a purinergic receptor antagonist that is known to potently suppresses hemichannel activity without great influence on GJIC [37], attenuated the calcium-depletion-elicited reduction in pan-cadherin and extracellular release of ATP and GSH, in a way similar to lindane and heptanol.

Oxidative stress is one of the major mechanisms behind the disassembly of cell junctions [22,44,52,53], which underlies many

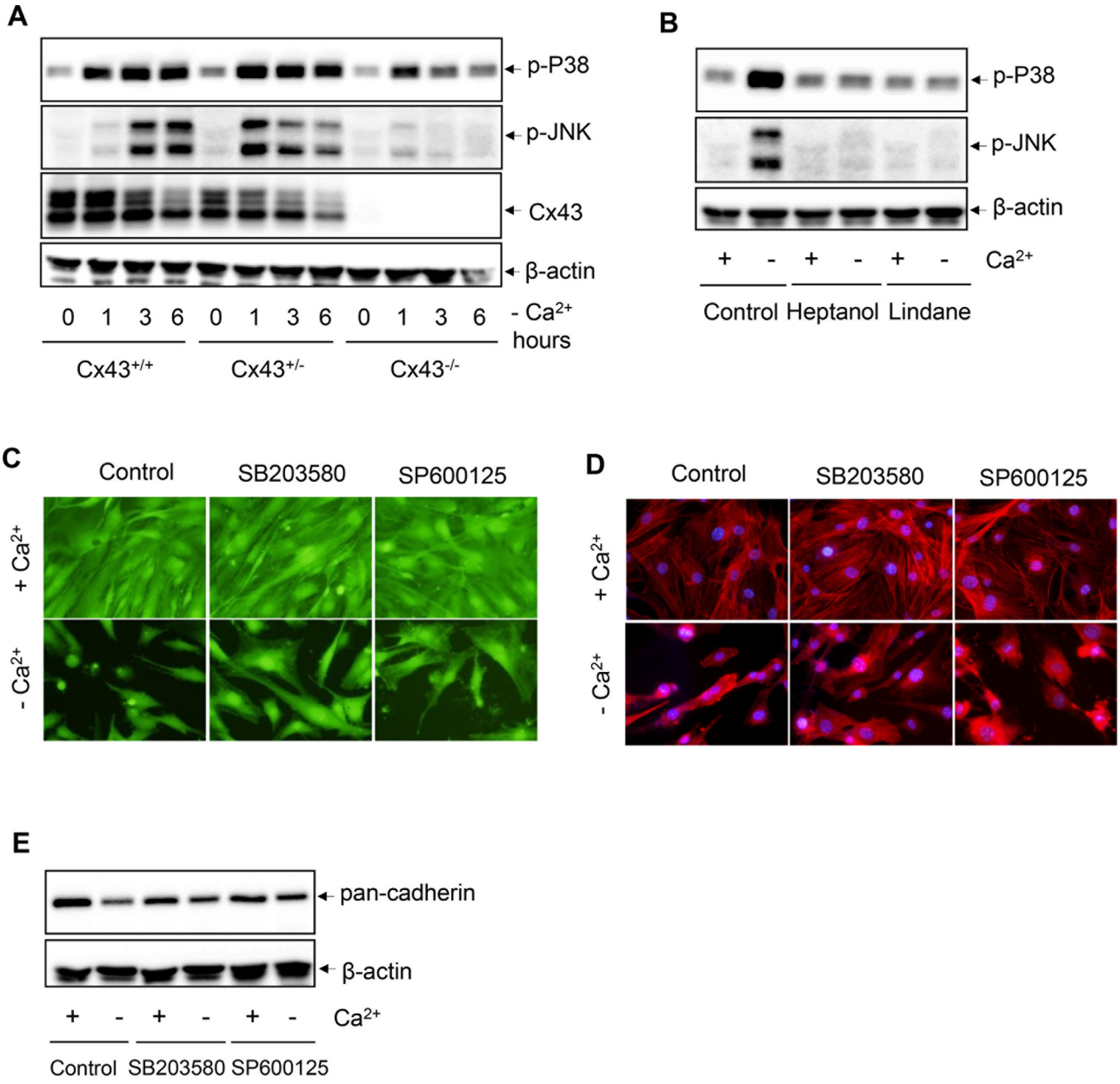

Fig. 5. P38 and JNK mediate hemichannel-induced cell shape change and disassembly of AJs. (A, B) Influence of Cx43 channels on Ca^{2+} depletion-induced phosphorylation of P38 and JNK. (A) Cells were exposed to Ca^{2+}-free medium for the indicated time intervals. Cellular lysates were subjected to Western blot analysis for phosphorylated P38 and JNK. (B) Cells were exposed to Ca^{2+}-free medium for 3 h in the presence or absence of 3 mM Heptanol or 100 μM Lindane. Cellular protein was immunoblotted for phosphorylated P38 and JNK. (C-E) Influence of p38 and JNK on Ca^{2+} depletion-elicited changes in cell shape, cytoskeleton and pan-cadherin level. (C) $Cx43^{+/+}$ fibroblasts exposed to Ca^{2+}-free medium in the presence or absence of 25 μM SB203580 or 25 μM SP600125 for 24 h. Cells were then loaded with 100 nM calcein for 1 h and photographed. (D, E) $Cx43^{+/+}$ fibroblasts were treated the same as above for 10 h and 3 h, respectively. Cells were then subjected to phalloidin for F-actin (D) and Western blot analysis for pan-cadherin (E).

types of barrier dysfunction in various tissue and organs under a wide range of pathological situations, including ischemia, inflammation and metabolic inhibition. In this study, the disassembly of cell junctions was preceded by the occurrence of oxidative stress, as indicated by the elevated level of protein carbonyl modification and activation of oxidative sensitive kinases, P38 and JNK. Furthermore, inhibition of oxidative stress limited the dysfunction of cell junctions. Our observations thus pointed to a causative role of oxidative stress in the disruption of cell junctions under Ca^{2+} -free condition. Intriguingly, inhibition of Cx channels with chemical inhibitor or Cx43 siRNA affected cell junctions and the related molecular events in a way similar to the antioxidants, implying that it might be work through modification of intracellular redox status.

The question naturally occurs as to how hemichannels influenced intracellular redox status. One possible explanation was that activation of hemichannels led to the extracellular loss of small molecules that are indispensable for the maintenance of intracellular redox status, such as GSH, ATP and NAD^+ [15,18,19]. In this study, GSH might play a key role. As a major oxygen radical scavenger, GSH provides the first line of defense against oxidative stress and cell injury. The loss of GSH through hemichannels, as demonstrated in this study and several previous reports [15,18,54], may weaken the cellular defense against oxidative stress. In agreement with this notion, $Cx43^{+/+}$ fibroblasts exhibited severer oxidative stress than $Cx43^{-/-}$ cells. In addition, supplement of cells with GSH indeed attenuated the stress-elicited modification of protein carbonyls and activation of oxidative sensitive kinases. Besides GSH, hemichannel-

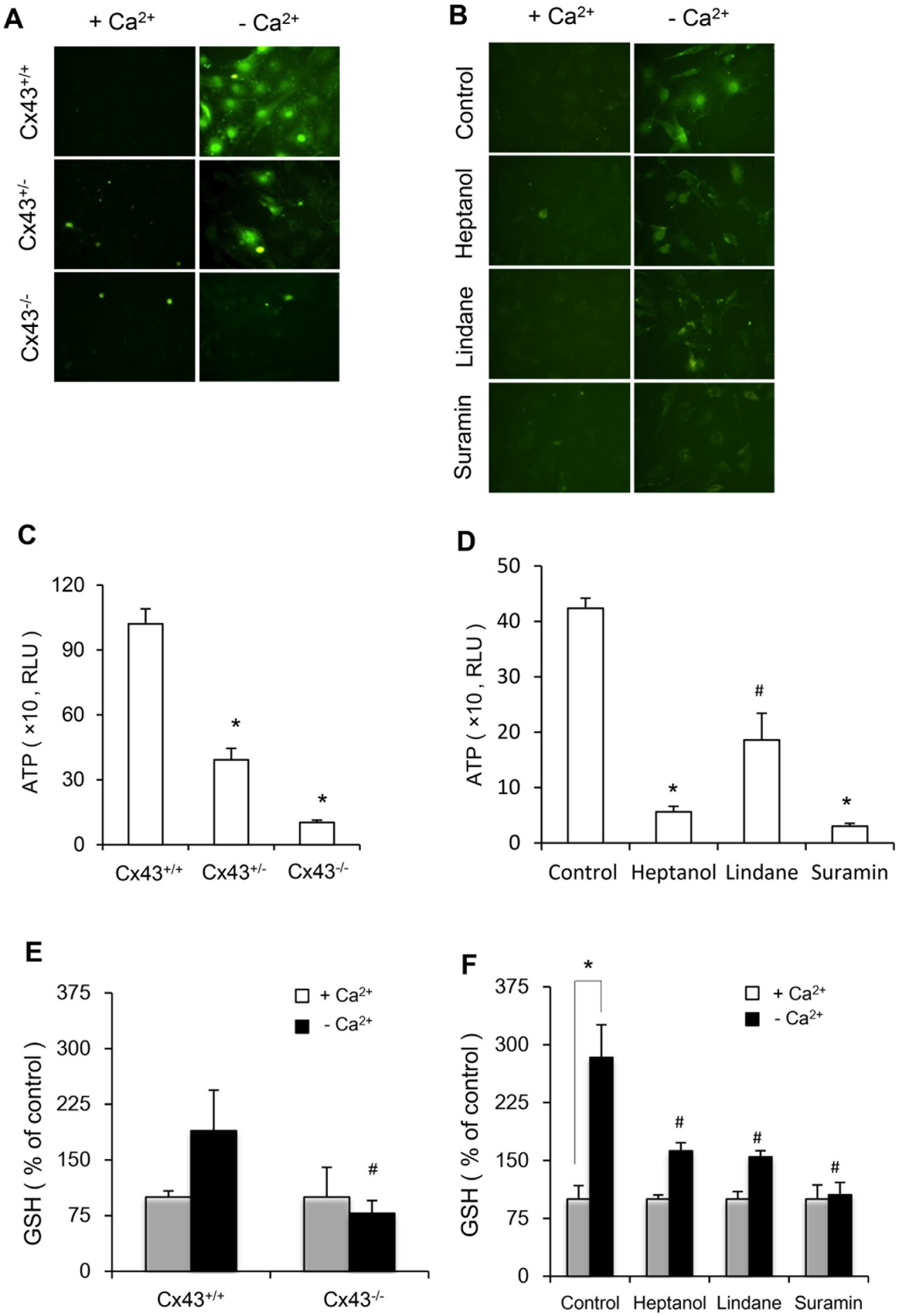

Fig. 6. Removal of extracellular Ca^{2+} activates Cx43 hemichannels and causes extracellular release of GSH. (A) Difference in LY uptake among $Cx43^{+/+}$, $Cx43^{+/-}$ and $Cx43^{-/-}$ fibroblasts. Fibroblasts were exposed to either normal or Ca^{2+}-free medium that contained 0.1% LY for 15 min. The cellular uptake of LY was photographed. (B) $Cx43^{+/+}$ fibroblasts were pre-treated with or without 3 mM heptanol, 100 μM lindane, or 200 μM suramin for 20 min. Cells were then exposed to normal or Ca^{2+}-free medium that contained 0.1% LY for an additional 15 min (C) Difference in ATP release among $Cx43^{+/+}$, $Cx43^{+/-}$ and $Cx43^{-/-}$ fibroblasts. Fibroblasts were exposed to either normal or Ca^{2+}-free medium for 15 min. ATP activity in culture supernatants was measured. Results shown are mean ± SE (n=5;* $P<0.01$). (D) $Cx43^{+/+}$ fibroblasts were pre-treated with or without 3 mM heptanol, 100 μM lindane, or 200 μM suramin for 20 min. Cells were then exposed to normal or Ca^{2+}-free medium for 15 min. Supernatants were collected and assayed for ATP activity. Results shown are mean ± SE (n=4;* $P<0.01$; # $P<0.05$). (E) Difference in GSH release between $Cx43^{+/+}$ and $Cx43^{-/-}$ fibroblasts. Fibroblasts were exposed to normal or Ca^{2+}-free medium for 30 min (F) $Cx43^{+/+}$ fibroblasts were pre-treated with or without 3 mM heptanol, 100 μM lindane, or 200 μM suramin for 20 min. Cells were then exposed to normal or Ca^{2+}-free medium for 30 min. GSH in culture supernatants were measured. Results are expressed as percentage of normal Ca^{2+} control (mean ± SE; n=4;* $P<0.01$; # $P<0.05$ vs. Ca^{2+}-free control).

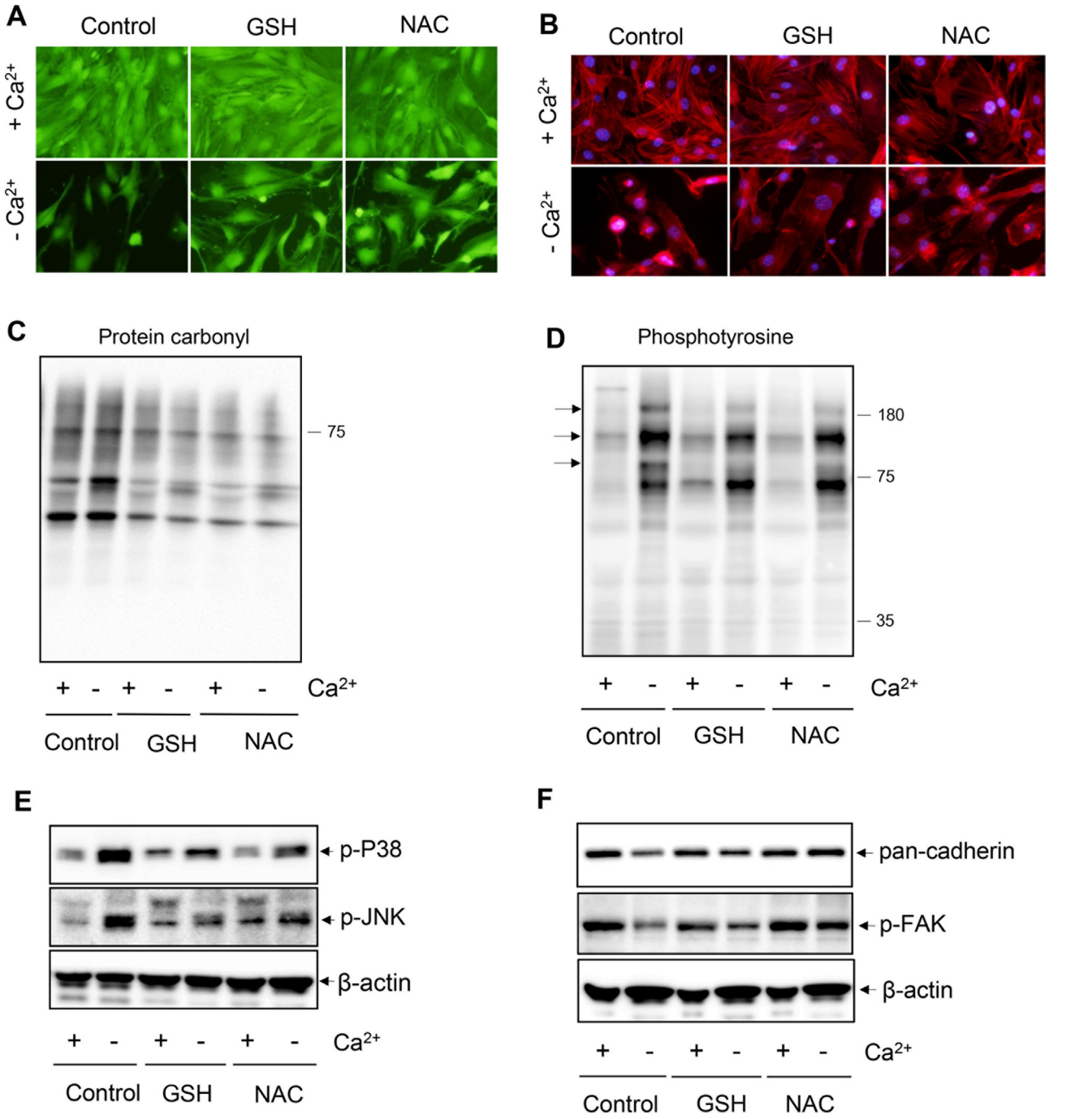

Fig. 7. Supplement of cells with GSH counteracts Ca^{2+} deprivation-elicited changes in cell shape and related molecular events. (A-B) Effect of exogenous GSH on Ca^{2+} depletion-induced changes in cell shape and cytoskeleton organization. (A) $Cx43^{+/+}$ fibroblasts exposed to Ca^{2+}-free medium in the presence or absence of 2 mM GSH or NAC for 24 h. Cells were then loaded with 100 nM calcein for 1 h and photographed. (B) $Cx43^{+/+}$ fibroblasts were treated the same as above for 10 h and subjected to phalloidin for F-actin. (C-F) Influence of GSH on Ca^{2+} depletion-induced changes in structural and functional proteins. $Cx43^{+/+}$ fibroblasts pre-treated with 2 mM GSH and 2 mM NAC for 20 min before exposed to the Ca^{2+}-free medium for 3 h (C, D, F) or 1 h (E). Cellular lysates were subjected to western blot analysis for carbonyl groups (C), tyrosine phosphorylation (D), P38 and JNK (E), as well as Pan-cadherin and p-FAK (F).

derived ATP could also contribute to the altered redox status. We have recently documented that extracellular ATP derived from hemichannels negatively regulated AMPK, an energy sensor that has anti-oxidative property [19] and has been reported to regulate both disassembly and assembly of TJs in epithelial cells [55].

It is worth mentioning that intercellular GJs also regulate intracellular redox status. Previous reports have shown that GJs promoted oxidative cell injury through intercellular transmission of Ca^{2+} and free radicals [56,57]. In addition, Cx43 also regulates intracellular oxidative status through communication-independent mechanisms. We have reported a potential implication of Cx43 in the regulation of NADPH oxidase [31], an enzyme that catalyzes the production of superoxide from oxygen and NADPH. In addition, we have recently identified thioredoxin-interacting protein (TXNIP) as a target of Cx43, contributing to the regulatory effects of Cx43 on oxidative drug injury [29]. Interestingly, both NADHP oxidase and TXNIP have been reported to be involved in the disassembly of tight and adherens junctions [58,59]. Thus, regulation of these molecules by Cx channels could also contribute to the observed effects in this investigation.

The role of hemichannels in the disassembly of TJs might promote one to speculate that pannexin channels, which are similar to Cx hemichannels in structure and function [60], could also be involved in the control of TJs. Although this possibility cannot be excluded, it was less likely to occur in our experimental setting, because NRK cells used in this study was reported to be pannexin-deficient [61]. More detailed analysis using other cell types may help answer the question. It is also worth mentioning that several recent studies have characterized NLRP3 inflammasome as an underlying mechanism behind the disassembly of cell junctions in several pathological situations [62,63]. Given that intracellular redox status, TXNIP level, and hemichannel activity are all implicated in the regulation of NLRP3 activation and inflammasome formation [60,63], involvement of this mechanism in our experimental system is highly probable. This hypothesis needs to be validated in the future.

In conclusion, we demonstrated that Cx43 channels contribute to low Ca^{2+}-elicited disassembly of cell junctions through regulation of cellular oxidative status. Our study thus characterized Cx43 channel as a presently unrecognized factor implicated in the regulation of cell junctions. Given many pathological stimuli that disrupt cell junctions also activate hemichannels, targeting hemichannels could be developed to limit the loss of barrier integrity.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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References

[1] J.M. Anderson, C.M. Van Itallie, Physiology and function of the tight junction, Cold Spring Harb. Perspect. Biol. 1 (2) (2009) a002584.
[2] E.E. Schneeberger, R.D. Lynch, The tight junction: a multifunctional complex, Am. J. Physiol. Cell Physiol. 286 (6) (2004) C1213–C1228.
[3] J.C. Saez, V.M. Berthoud, M.C. Branes, A.D. Martinez, E.C. Beyer, Plasma membrane channels formed by connexins: their regulation and functions, Physiol. Rev. 83 (4) (2003) 1359–1400.
[4] N. Sawada, M. Murata, K. Kikuchi, M. Osanai, H. Tobioka, T. Kojima, H. Chiba, Tight junctions and human diseases, Med. Electron Microsc. 36 (3) (2003) 147–156.
[5] O. Tornavaca, M. Chia, N. Dufton, L.O. Almagro, D.E. Conway, A.M. Randi, M. A. Schwartz, K. Matter, M.S. Balda, ZO-1 controls endothelial adherens junctions, cell-cell tension, angiogenesis, and barrier formation, J. Cell Biol. 208 (6) (2015) 821–838.
[6] B.N. Giepmans, W.H. Moolenaar, The gap junction protein connexin43 interacts with the second PDZ domain of the zona occludens-1 protein, Curr. Biol. 8 (16) (1998) 931–934.
[7] B.N. Giepmans, Gap junctions and connexin-interacting proteins, Cardiovasc. Res. 62 (2) (2004) 233–245.
[8] L. González-Mariscal, R. Tapia, D. Chamorro, Crosstalk of tight junction components with signaling pathways, Biochim Biophys. Acta 1778 (3) (2008) 729–756.
[9] J.A. Tunggal, I. Helfrich, A. Schmitz, H. Schwarz, D. Günzel, M. Fromm, R. Kemler, T. Krieg, C.M. Niessen, E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions, EMBO J. 24 (6) (2005) 1146–1156.
[10] Y. Liu, A. Nusrat, F.J. Schnell, T.A. Reaves, S. Walsh, M. Pochet, C.A. Parkos, Human junction adhesion molecule regulates tight junction resealing in epithelia, J. Cell Sci. 113 (13) (2000) 2363–2374.
[11] E.M. Frenzel, R.G. Johnson, Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin, Dev. Biol. 179 (1) (1996) 1–16.
[12] R.A. Meyer, D.W. Laird, J.-P. Revel, R.G. Johnson, Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies, J. Cell Biol. 119 (1) (1992) 179–189.
[13] Z. Chai, D.A. Goodenough, D.L. Paul, Cx50 requires an intact PDZ-binding motif and ZO-1 for the formation of functional intercellular channels, Mol. Biol. Cell 22 (23) (2011) 4503–4512.
[14] J. Yao, T. Oite, M. Kitamura, Gap junctional intercellular communication in the juxtaglomerular apparatus, Am. J. Physiol. Ren. Physiol. 296 (5) (2009) F939–F946.
[15] X. Fang, T. Huang, Y. Zhu, Q. Yan, Y. Chi, J.X. Jiang, P. Wang, H. Matsue, M. Kitamura, J. Yao, Connexin43 hemichannels contribute to cadmium-induced oxidative stress and cell injury, Antioxid. Redox Signal 14 (12) (2011) 2427–2439.
[16] S. Bruzzone, L. Guida, E. Zocchi, L. Franco, A. De Flora, Connexin 43 hemi channels mediate Ca2+-regulated transmembrane NAD+ fluxes in intact cells, FASEB J. 15 (1) (2001) 10–12.
[17] H.-B. Zhao, N. Yu, C.R. Fleming, Gap junctional hemichannel-mediated ATP release and hearing controls in the inner ear, Proc. Natl. Acad. Sci. USA 102 (51) (2005) 18724–18729.
[18] M.H. Stridh, M. Tranberg, S.G. Weber, F. Blomstrand, M. Sandberg, Stimulated efflux of amino acids and glutathione from cultured hippocampal slices by omission of extracellular calcium like involvement of connexin hemichannels, J. Biol. Chem. 283 (16) (2008) 10347–10356.
[19] Y. Chi, K. Gao, K. Li, S. Nakajima, S. Kira, M. Takeda, J. Yao, Purinergic control of AMPK activation by ATP released through connexin 43 hemichannels–pivotal roles in hemichannel-mediated cell injury, J. Cell Sci. 127 (7) (2014) 1487–1499.
[20] D.W. Laird, The gap junction proteome and its relationship to disease, Trends Cell Biol. 20 (2) (2010) 92–101.
[21] S. Basuroy, A. Seth, B. Elias, A.P. Naren, R. Rao, MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide, Biochem. J. 393 (Pt 1) (2006) 69–77.
[22] S. Basuroy, P. Sheth, D. Kuppuswamy, S. Balasubramanian, R.M. Ray, R.K. Rao, Expression of kinase-inactive c-Src delays oxidative stress-induced disassembly and accelerates calcium-mediated reassembly of tight junctions in the Caco-2 cell monolayer, J. Biol. Chem 278 (14) (2003) 11916–11924.
[23] P.V. Usatyuk, S. Vepa, T. Watkins, D. He, N.L. Parinandi, V. Natarajan, Redox regulation of reactive oxygen species-induced p38 MAP kinase activation and barrier dysfunction in lung microvascular endothelial cells, Antioxid. Redox Signal. 5 (6) (2003) 723–730.
[24] L. Vergara, X. Bao, E. Bello-Reuss, L. Reuss, Do connexin 43 gap-junctional hemichannels activate and cause cell damage during ATP depletion of renal-tubule cells? Acta Physiol. Scand. 179 (1) (2003) 33–38.
[25] M.A. Retamal, K.A. Schalper, K.F. Shoji, M.V. Bennett, J.C. Sáez, Opening of connexin 43 hemichannels is increased by lowering intracellular redox potential, Proc. Natl. Acad. Sci. USA 104 (20) (2007) 8322–8327.
[26] M.A. Retamal, Connexin and Pannexin hemichannels are regulated by redox potential, Front Physiol. 5 (2014).
[27] J.A. Orellana, P.J. Sáez, K.F. Shoji, K.A. Schalper, N. Palacios–Prado, V. Velarde, C. Giaume, M.V. Bennett, J.C. Sáez, Modulation of brain hemichannels and gap junction channels by pro-inflammatory agents and their possible role in neurodegeneration, Antioxid. Redox Signal 11 (2) (2009) 369–399.
[28] J.E. Contreras, H.A. Sánchez, E.A. Eugenín, D. Speidel, M. Theis, K. Willecke, F. F. Bukauskas, M.V. Bennett, J.C. Sáez, Metabolic inhibition induces opening of unapposed connexin 43 gap junction hemichannels and reduces gap junctional communication in cortical astrocytes in culture, Proc. Natl. Acad. Sci. USA 99 (1) (2002) 495–500.
[29] K. Gao, Y. Chi, X. Zhang, H. Zhang, G. Li, W. Sun, M. Takeda, J. Yao, A novel TXNIP-based mechanism for Cx43–mediated regulation of oxidative drug injury, J. Cell Mol. Med 19 (10) (2015) 2469–2480.
[30] H.T. Le, W.C. Sin, S. Lozinsky, J. Bechberger, J.L. Vega, X.Q. Guo, J.C. Sáez, C. C. Naus, Gap junction intercellular communication mediated by connexin43 in astrocytes is essential for their resistance to oxidative stress, J. Biol. Chem. 289 (3) (2014) 1345–1354.
[31] Q. Yan, K. Gao, Y. Chi, K. Li, Y. Zhu, Y. Wan, W. Sun, H. Matsue, M. Kitamura, J. Yao, NADPH oxidase-mediated upregulation of connexin43 contributes to podocyte injury, Free Radic. Biol. Med 53 (6) (2012) 1286–1297.
[32] P.V. Usatyuk, N.L. Parinandi, V. Natarajan, Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins, J. Biol. Chem. 281 (46) (2006) 35554–35566.
[33] H. Lum, K.A. Roebuck, Oxidant stress and endothelial cell dysfunction, Am. J. Physiol. Cell Physiol. 280 (4) (2001) C719–C741.
[34] G. Schreibelt, G. Kooij, A. Reijerkerk, R. van Doorn, S.I. Gringhuis, S. van der Pol, B.B. Weksler, I.A. Romero, P.O. Couraud, J. Piontek, I.E. Blasig, C.D. Dijkstra, E. Ronken, H.E. de Vries, Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling, FASEB J. 21 (13) (2007) 3666–3676.
[35] C.M. Zehendner, L. Librizzi, J. Hedrich, N.M. Bauer, E.A. Angamo, M. de Curtis, H.J. Luhmann, Moderate hypoxia followed by reoxygenation results in blood-brain barrier breakdown via oxidative stress-dependent tight-junction protein disruption, PLoS One 8 (12) (2013) e82823.
[36] T. Huang, Y. Zhu, X. Fang, Y. Chi, M. Kitamura, J. Yao, Gap junctions sensitize cancer cells to proteasome inhibitor MG132–induced apoptosis, Cancer Sci. 101 (3) (2010) 713–721.
[37] Y. Chi, K. Gao, H. Zhang, M. Takeda, J. Yao, Suppression of cell membrane permeability by suramin: involvement of its inhibitory actions on connexin 43 hemichannels, Br. J. Pharm. 171 (14) (2014) 3448–3462.

[38] J. Yao, T. Huang, X. Fang, Y. Chi, Y. Zhu, Y. Wan, H. Matsue, M. Kitamura, Disruption of gap junctions attenuates aminoglycoside-elicited renal tubular cell injury, Br. J. Pharm. 160 (8) (2010), 2055-68.
[39] K. Li, Y. Chi, K. Gao, Q. Yan, H. Matsue, M. Takeda, M. Kitamura, J. Yao, Connexin43 hemichannel-mediated regulation of connexin43, PloS One 8 (2) (2013) e58057.
[40] T.N. Meyer, C. Schwesinger, J. Ye, B.M. Denker, S.K. Nigam, Reassembly of the tight junction after oxidative stress depends on tyrosine kinase activity, J. Biol. Chem. 276 (25) (2001) 22048–22055.
[41] C.B. Collares-Buzato, M.A. Jepson, N.L. Simmons, B.H. Hirst, Increased tyrosine phosphorylation causes redistribution of adherens junction and tight junction proteins and perturbs paracellular barrier function in MDCK epithelia, Eur. J. Cell Biol. 76 (2) (1998) 85–92.
[42] J.M. Staddon, K. Herrenknecht, C. Smales, L.L. Rubin, Evidence that tyrosine phosphorylation may increase tight junction permeability, J. Cell Sci. 108 (2) (1995) 609–619.
[43] P. Sheth, S. Basuroy, C. Li, A.P. Naren, R.K. Rao, Role of phosphatidylinositol 3-kinase in oxidative stress-induced disruption of tight junctions, J. Biol. Chem. 278 (49) (2003) 49239–49245.
[44] R. Rao, Oxidative stress-induced disruption of epithelial and endothelial tight junctions, Front. Biosci. 13 (2008) 7210.
[45] E. Herlaar, Z. Brown, p38 MAPK signalling cascades in inflammatory disease, Mol. Med. Today 5 (10) (1999) 439–447.
[46] W.-y Lui, W.M. Lee, C.Y. Cheng, Transforming growth factor β3 regulates the dynamics of Sertoli cell tight junctions via the p38 mitogen-activated protein kinase pathway, Biol. Reprod. 68 (5) (2003) 1597–1612.
[47] T. Oshima, H. Miwa, T. Joh, Aspirin induces gastric epithelial barrier dysfunction by activating p38 MAPK via claudin-7, Am. J. Physiol. Cell Physiol. 295 (3) (2008) C800–C806.
[48] L. Zhang, F. Jouret, J. Rinehart, J. Sfakianos, I. Mellman, R.P. Lifton, L.H. Young, M.J. Caplan, AMP-activated protein kinase (AMPK) activation and glycogen synthase kinase-3β (GSK-3β) inhibition induce Ca2+-independent deposition of tight junction components at the plasma membrane, J. Biol. Chem 286 (19) (2011) 16879–16890.
[49] D.J. Muller, G.M. Hand, A. Engel, G.E. Sosinsky, Conformational changes in surface structures of isolated connexin 26 gap junctions, EMBO J. 21 (14) (2002) 3598–3607.
[50] B.R. Takens-Kwak, H.J. Jongsma, M.B. Rook, A.C. Van Ginneken, Mechanism of heptanol-induced uncoupling of cardiac gap junctions: a perforated patch-clamp study, Am. J. Physiol. 262 (6 Pt 1) (1992) C1531-8.
[51] R.L. Caruso, B.L. Upham, C. Harris, J.E. Trosko, Biphasic lindane-induced oxidation of glutathione and inhibition of gap junctions in myometrial cells, Toxicol. Sci. 86 (2) (2005) 417–426.
[52] J. Haorah, S.H. Ramirez, K. Schall, D. Smith, R. Pandya, Y. Persidsky, Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction, J. Neurochem 101 (2) (2007) 566–576.
[53] T.A. Bailey, N. Kanuga, I.A. Romero, J. Greenwood, P.J. Luthert, M.E. Cheetham, Oxidative Stress Affects the Junctional Integrity of Retinal Pigment Epithelial Cells, Investig. Opthalmol. Visual Sci. 45 (2) (2004) 675.
[54] S. Rana, R. Dringen, Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes, Neurosci. Lett. 415 (1) (2007) 45–48.
[55] B. Zheng, L.C. Cantley, Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase, Proc. Natl. Acad. Sci. USA 104 (3) (2007) 819–822.
[56] E.I. Azzam, S.M. de Toledo, J.B. Little, Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells, Proc. Natl. Acad. Sci. USA 98 (2) (2001) 473–478.
[57] V.A. Krutovskikh, C. Piccoli, H. Yamasaki, Gap junction intercellular communication propagates cell death in cancerous cells, Oncogene 21 (13) (2002) 1989–1999.
[58] K.D. Rochfort, L.E. Collins, R.P. Murphy, P.M. Cummins, Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions, PLoS One 9 (7) (2014) e101815.
[59] L.P. Singh, Thioredoxin interacting protein (TXNIP) and pathogenesis of diabetic retinopathy, J. Clin. Exp. Ophthalmol. 4 (2013).
[60] H.P. Makarenkova, V.I. Shestopalov, The role of pannexin hemichannels in inflammation and regeneration, Front Physiol. 5 (2014) 63.
[61] S. Penuela, R. Bhalla, X.Q. Gong, K.N. Cowan, S.J. Celetti, B.J. Cowan, D. Bai, Q. Shao, D.W. Laird, Pannexin 1 and pannexin 3 are glycoproteins that exhibit many distinct characteristics from the connexin family of gap junction proteins, J. Cell Sci. 120 (21) (2007) 3772–3783.
[62] Y. Zhuang, C. Hu, G. Ding, Y. Zhang, S. Huang, Z. Jia, A. Zhang, Albumin impairs renal tubular tight junctions via targeting the NLRP3 inflammasome, Am. J. Physiol. Ren. Physiol. 308 (9) (2015) F1012–F1019.
[63] A. Abderrazak, T. Syrovets, D. Couchie, K. El Hadri, B. Friguet, T. Simmet, M. Rouis, NLRP3 inflammasome: from a danger signal sensor to a regulatory node of oxidative stress and inflammatory diseases, Redox Biol. 4 (2015) 296–307.