Inhibition of connexin43 dephosphorylation is involved in protective effects of diltiazem on cardiac function during hypoxic injury

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Summary. Background: Connexin43 (Cx43), a gap junction protein, mediates cell-cell communication via electrical and chemical coupling. Ischemic stress of the cardiac muscle interrupts intercellular communication by changing the distribution and phosphorylation status of Cx43. This may be a factor contributing to reentrant arrhythmia. The calcium channel blocker diltiazem is known for its protective and anti-arrhythmogenic effect in ischemic heart disease. In this study, we assess the effect of diltiazem pretreatment upon ischemia-induced phosphorylation change of Cx43

Methods: Langendorff preparations of isolated Wistar rat hearts were performed. After stabilization, hearts were treated with (D+) or without diltiazem (D-), then subjected to hypoxia-reoxygenation. After perfusion, the left ventricle was prepared for immunocytochemistry and immunoblot analysis.

Results: During perfusion, left ventricular function was better in the D+ group than the D- group. Immunostaining of the heart indicated that dephosphorylated Cx43 (dpCx43) signal was increased after hypoxic perfusion, and this finding was confirmed by immunoblot data. The quantitative area analysis of dpCx43 using the immunohistochemical approach showed that the dpCx43-positive area was enlarged, as the hypoxic perfusion time was longer, and it was reduced by pretreatment of diltiazem. There was a negative correlation between the dpCx43 area and %RPP (rate-pressure product), calculated by heart rate and contraction force.

Conclusions: Pretreatment of diltiazem could protect the heart against hypoxia-reoxygenation injury by attenuation of dephosphorylation of Cx43. The anti-arrhythmic mechanism of diltiazem may include the preservation of phosphorylation status of Cx43 after hypoxia-reoxygenation injury.

Key words: Connexin 43, Diltiazem, Phosphorylation, Arrhythmia, Langendorff

Introduction

Arrhythmia after acute myocardial injury or cardiac surgery is known to be related to ischemia–reperfusion injury. Alteration of gap junction property is considered to be one of the pivotal mechanisms which cause re-entrance arrhythmia (Peters et al., 1997). Gap-junctions, which provide electrical and mechanical coupling between neighboring cells, are composed of connexin proteins and permit the direct exchange of cytoplasmic ions and small molecules of less than 1 kDa. In the connexin family, Connexin43 (Cx43) is the most abundant, and the principal protein for ventricular electrical coupling (Reaume et al., 1995; Ya et al., 1998; Gutstein et al., 2001).

A change in the phosphorylation status of Cx43 protein has been implicated in the regulation of channel functions involved in cellular homeostasis, electrical coupling, embryogenesis and the regulation of proliferation (Laird et al., 1991; Musil et al., 1991; Oelze et al., 1995; TenBroek et al., 2001; Leykauf et al., 2003). The alteration of the phosphorylation status of Cx43 is induced by cellular stresses, e.g. ischemia or hypoxia (Beardslee et al., 2000; Turner et al., 2004; Matsushita et al., 2006). Cx43 is phosphorylated at multiple serine residues in its c-terminal (Musil et al., 1991; Beardslee et al., 2000). In particular, the phosphorylation status of

serine 368 (Ser368) has been reported to play a central role for channel properties (Laird et al., 1991; Musil et al., 1991; Oelze et al., 1995; Beardslee et al., 2000; TenBroek et al., 2001; Leykauf et al., 2003).

Diltiazem, an L-type calcium channel blocker, is known to protect the heart from ischemia-reperfusion injury, including reduction of the infarct area (Weishaar et al., 1979; Bush et al., 1981; Zamanis et al., 1982). Diltiazem is also known for its anti-arrhythmic effect of reducing the risk of ventricular arrhythmia by slowing antegrade conduction velocity of atrio-ventricular node via inhibition of calcium influx into the cell (van Gils et al., 1986). However, the underlying effect of diltiazem on gap junction property is still unclear.

In this study, we evaluated the relationship between the effect of diltiazem mediated cardiac protection and the gap junction protein by using an ex-vivo perfusion system of rat heart.

Materials and methods

Preparation and perfusion procedure

All the procedures performed on laboratory animals were approved by the institutional animal care and committee of Juntendo University School of Medicine and all the animal experiments were carried out in compliance with the guidelines for animal experimentation of Juntendo University School of Medicine.

Hearts were isolated from male Wistar rats (280-360g), the ascending aorta was cannulated, and subjected to a constant flow (13 ml/minutes) Langendorff apparatus as previously described (Okada et al., 2000; Matsushita et al., 2006). After stabilization for 20 min for all groups, the hearts for the diltiazem-pretreated group (D+: n=8 in control and 20 min-hypoxia groups, and n=7 in 40 min-hypoxia group, respectively) were perfused for 10 min with 1 µmol/L of diltiazem (Sigma-Aldrich; St. Louise, MO) contained in perfusate. After the diltiazem treatment, for the hypoxia group, the heart was perfused with hypoxic solution (glucose replaced with sucrose) for 20 or 40 min, then subjected to 30 min reoxygenation. Zero min-hypoxia group (control hearts, n=8 each) were submitted to 30 min of oxygenated perfusion following the stabilizing period or diltiazem treatment. Coronary effluent was collected to measure the amount of glutamic oxaloacetic transaminase (GOT) for an index of non-reversible cardiac damage

Immunofluorescence

At the end of the Langendorff experiments, hearts were injected with 4% paraformaldehyde (PFA) solution through the aortic cannula. The left ventricle was then trimmed to include papillary muscle, and the cardiac pieces were immersed in PFA for 30 min for fixation, as described previously (Matsushita et al., 2006). Three

kinds of primary antibodies that recognized differential phosphorylation status against Cx43 antibody were used. (i) anti-Cx43 antibodies which recognized both phosphorylated and dephosphorylated Cx43 (t-Cx43, Sigma-Aldrich), (ii) anti-phospho-Cx43 antibody which recognized only phosphorylation form Cx43 at Ser368 (pCx43, Cell-Signaling Technology; Beverly, MA) and (iii) anti-dephospho-Cx43 antibody which recognized only dephosphorylation form Cx43 at Ser368 of (dpCx43, Zymed; San Francisco, CA). For details of primary antibodies, see reference Matsushita et al., 2006. TRITC or FITC conjugated antibodies (Jackson ImmunoResearch; West Grove, PA) were used as secondary antibodies.

Western blot analysis

Hearts were solubilized in PBS containing protease inhibitors, 1% SDS and 5 mM EDTA, electrophoresed on 12% polyacrylamide gels, then transferred to nitrocellulose membranes. Blots were incubated with primary and then HRP-conjugated secondary antibodies (BioRad; Hercules, CA). The signal was detected using the ECL Western Blotting Detection System (Amersham, Piscataway; NJ). Quantification of protein bands was done by densitometry with the use of QuantiScan software (Biosoft; Cambridge, United Kingdom).

Image analysis

Fluorescence specimens were observed with a Leica DMR microscope. Images were captured with a Hamamatsu Orca-ER CCD camera and analyzed using AquaCosmos software (Hamamatsu Photonics; Hamamatsu, Japan).

Statistical analysis

Statistical analysis was performed with the aid of commercially available software (Statcel 2; O.M.S. publisher, Saitama, Japan). Comparison of area analysis among groups was performed by Tukey-Kramer comparison test, the correlation analysis with scatter gram was performed by simple regression analysis.

Results

Physiological assessment of the heart

To determine the functional change of heart during perfusion, we recorded heart rate (HR), left ventricular developed pressure (LVDP) and left ventricular end-diastolic pressure (LVEDP). Rate-pressure product (RPP = HR x LVDP) was calculated as an index of cardiac work load. The values of each index at the end of the stabilization period were set as a baseline (100%). The time course changes of %HR, %DP, %RPP, %LVEDP and GOT on the reoxygenation group are shown in

*Diltiazem preserves Cx43 phosphorylation*

Figure 1.

When the hearts were perfused with hypoxia solution, both HR and LVDP immediately decreased and reached almost zero after 20 minutes hypoxia (Fig. 1a,b), then increased again upon reoxygenation. %HR returned back almost to baseline in all groups at the end of reoxygenation, even in the longer hypoxic time group (hypoxic time= 0 min: 105.4%±5.2, 20 min: 93.8%±5.6, 40 min: 100.5%±6.9, respectively [mean ± SE, p=NS]). %LVDP also recovered during reoxygenation, but incompletely. The level of %LVDP recovery was better in the shorter time group of hypoxic perfusion, although %HR was not affected by hypoxic time.

When the heart was pretreated with diltiazem (D+ group), %HR decreased to about 70% of the baseline, although %LVDP was preserved (Fig.1a-c). During hypoxia, both %HR and %LVDP were also depressed, although %LVDP in D+ group was consistently better than D-. In the reoxygenation period, %LVDP in D+ still showed a higher value than D-, although %HR was recovered almost to baseline value at the end of perfusion in both D- and D+ groups.

At the end of reoxygenation following 40 minutes hypoxia, the recovery of %RPP was markedly improved in the D+ group compared to D- (Fig 1c: 32.9%±5.9 vs.55.5%±6.0: D- vs. D+, respectively. p<0.05). In addition, the %RPP of 20 min-hypoxia group also tended to improve in the D+ group, although there was no statistical significant between D- and D+ (55.2%±13.4 vs. 69.3%±15.9, D- vs. D+, p=0.07).

LVEDP was adjusted to 0-3 mmHg during the stabilizing period by inflating a latex balloon. LVEDP was increased by hypoxia and reached plateau at around 10 minutes (Fig. 1d). At the end of the hypoxia period, this plateau value was 57.9±5.4 mmHg in 20 min-hypoxia and 60.1±3.8 mmHg in 40 min-hypoxia group, respectively. It was recovered by reoxygenation, although not completely back to baseline, and it showed a higher value in the longer hypoxia group at the end of reoxygenation (14.5±3.1 mmHg in 20 min-hypoxia and 31.9±4.2 mmHg in 40 min-hypoxia group, respectively). In contrast, pretreatment of diltiazem consistently reduced the LVEDP elevation in all hypoxia groups at all time points (20 min-hypoxia: 30.9±5.9, 40 min-hypoxia:
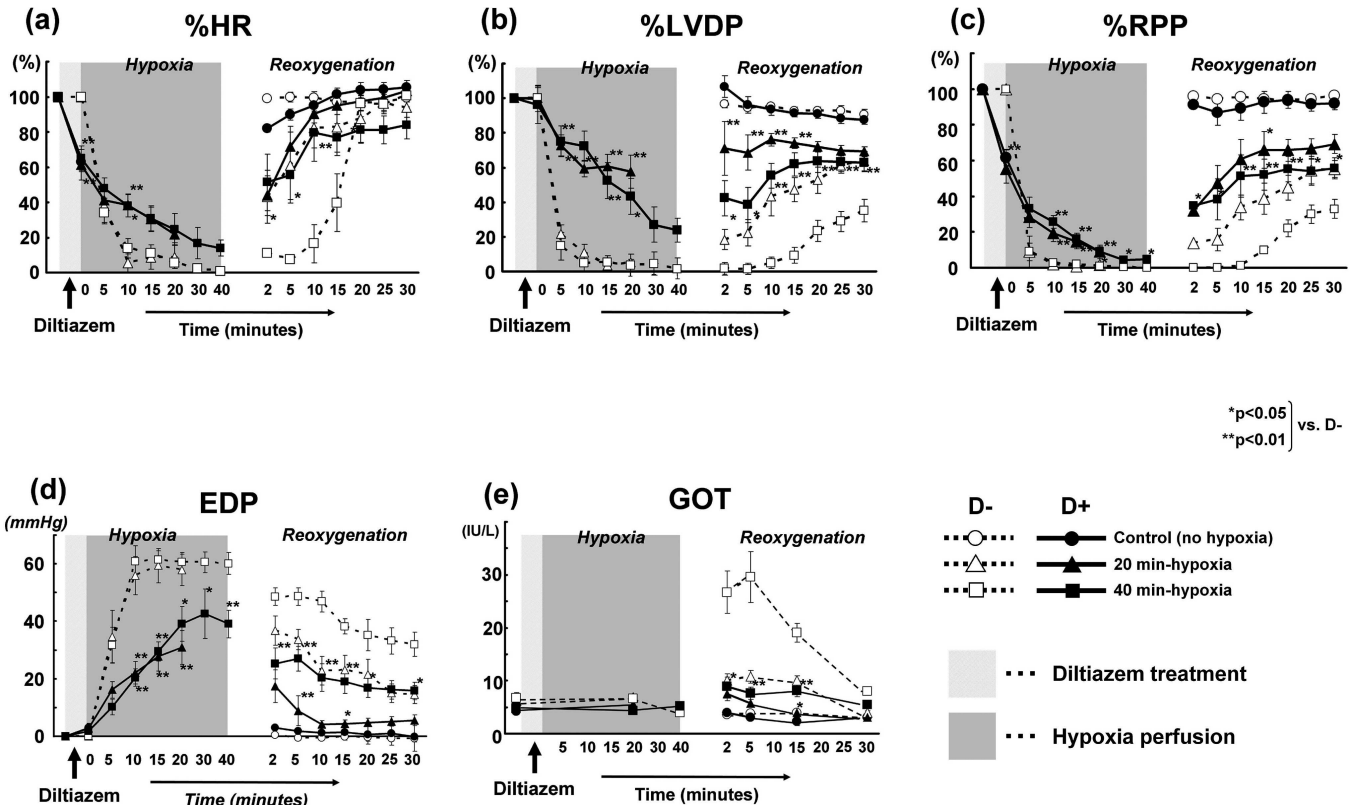
Fig. 1. The time course change of heart functions. **a.** In D- group, HR went down immediately after hypoxia perfusion started, then the heart stopped beating (dashed line). In D+, the HR was decreased when diltiazem was treated, however the heart preserved beating during hypoxia (solid line). %HR was recovered to baseline in all groups at the end of reoxygenation. **b.** Diltiazem pretreated heart also maintained the heart contractility both in hypoxia and reoxygenation period. **c.** During diltiazem treatment, %RPP dropped around 60%. However, the recovery of %RPP showed better in D+ group after reoxygenation. **d.** Pretreatment of diltiazem prevented LVEDP elevation. **e.** GOT in coronary effluent was measured. GOT release was dramatically increased at 2, 5 and 15 min of reoxygenation after 40 min-hypoxia in D- group and pretreatment of diltiazem markedly reduced it.
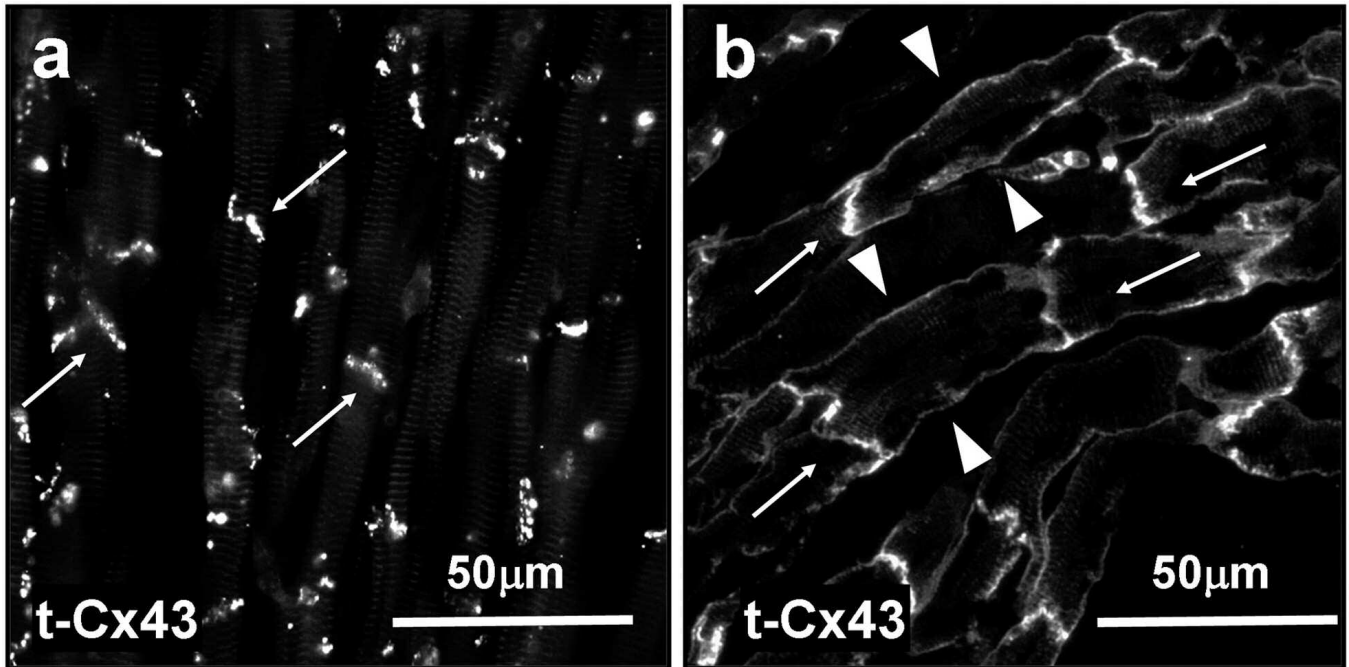
Fig. 2. Immunostaining with anti-tCx43 antibody which recognizes both pCx43 and dpCx43. **a.** Cx43 in control heart (0 min-hypoxia). They located virtually at intercalated disk. **b.** Heart tissue after exposing hypoxia for 40 minutes. Cx43 changed its location to entire plasma membrane. Interestingly, their distribution also altered from dots to diffuse. (Arrow indicates intercalated disk, arrowhead indicates migrated Cx43 around plasma membrane).
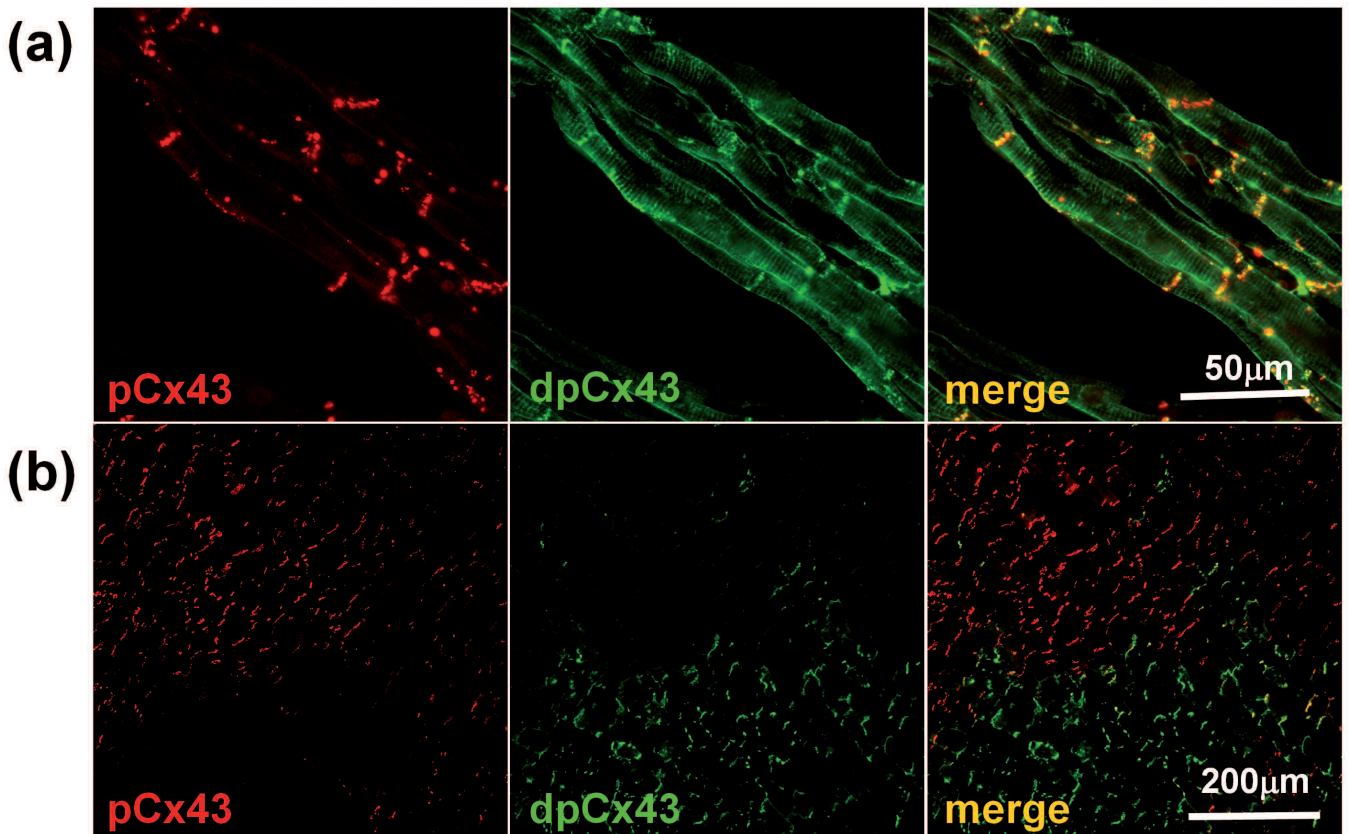
Fig. 3. Color images of phospho-specific Cx43 redistribution after exposing hypoxia. **a.** The signal around entire plasma membrane indicates only dephosphorylated Cx43 (green), and phosphorylated Cx43 (red) was seen only at intercalated disks. **b.** Lower magnification image of hypoxia heart. Phosphorylation change has happened not diffuse but patchy like island.

39.0±4.8 at the end of hypoxia perfusion, and 20 min-hypoxia: 5.5±1.8, 40 min-hypoxia: 15.8±3.0 mmHg at the end of reoxygenation, respectively).

In the 40 min-hypoxia group, GOT release was greatly increased at 2, 5 and 15 min after reoxygenation (R2, R5 and R15 minutes, respectively) compared with non-hypoxia control (Fig. 1e). In contrast, the GOT release at the early phase on reoxygenation was consistently attenuated in the D+ group (R2 min=26.6±4.6 vs. 8.9±1.7 IU/L, R5 min= 29.3±5.3 vs.

7.6±1.2 IU/L and R15 min=19.1±1.8 vs.8.1±1.1 IU/L: D- vs. D+ in 40 min-hypoxia, respectively). There was also a trend to an attenuation effect of diltiazem on GOT release in the 20 min-hypoxia group, although this did not reach statistical significance.

### Diltiazem preserves phosphorylation of connexin43

We performed immunostaining to clarify the localization of Cx43 and its phosphorylation status by
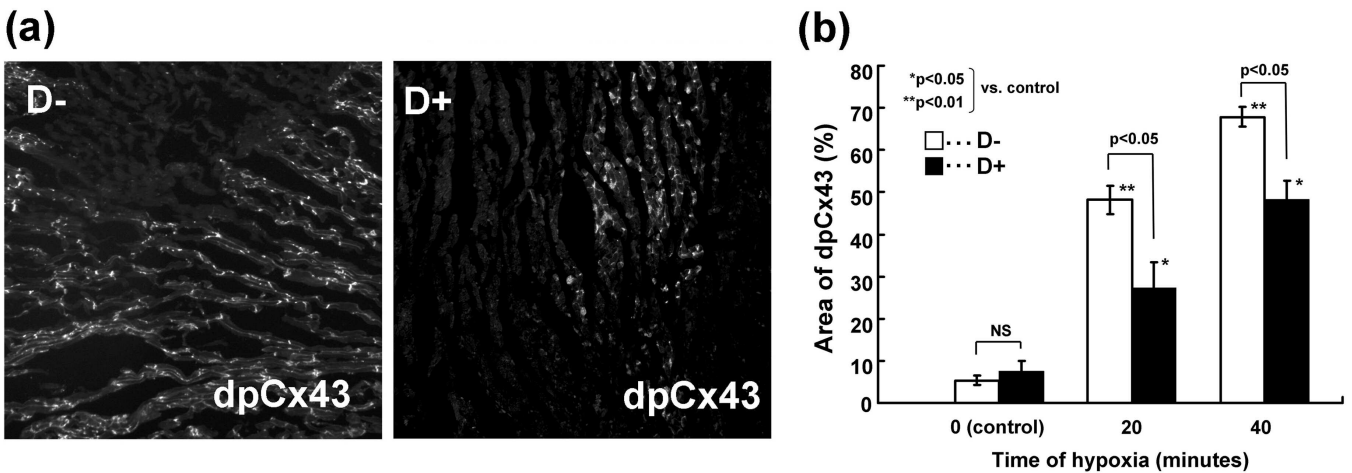
Fig. 4. a. An example image of the heart stained by dpCx43ab (40 minutes-hypoxia group). Dephosphorylation of Cx43 was less in diltiazem treated heart. **b.** Quantified area analysis of dpCx43. Cx43 was more dephosphorylated as hypoxia time was longer, however diltiazem attenuated the dephosphorylation of Cx43 ($p<0.05$ vs. D- group).
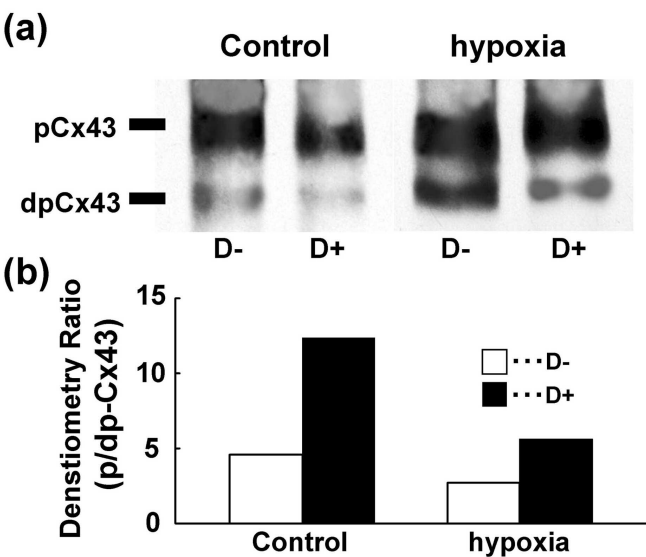
Fig. 5. Western blot analysis of phosphorylation change of Cx43. Correspondently with histochemical results, Cx43 was more dephosphorylated following hypoxia, and diltiazem attenuated dephosphorylation change and preserved phosphorylation of Cx43.
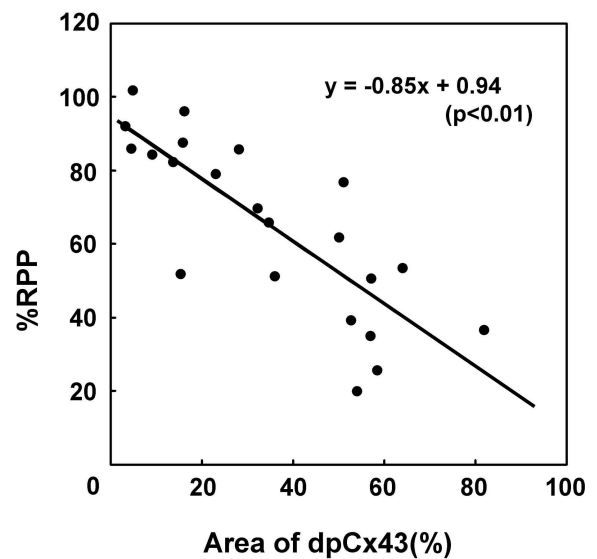
Fig. 6. Scatter gram showed that there was good correlation between heart function (%RPP) and dephosphorylation area of Cx43 (area of dpCx43). The slope was y=-0.85x+0.94 ($p<0.01$).

using three kinds of antibodies which recognized different phosphorylation status of Cx43. In the control heart, as has already been reported (Musil et al., 1991; Beardslee et al., 2000; Matsushita et al., 2006), Cx43 is predominantly located at the intercalated disk (Fig. 2a). When the heart was subjected to hypoxia, the signal of Cx43 was also seen around entire the plasma membrane (Fig. 2b). We performed double staining to assess the phospho-specific distribution of Cx43. Interestingly, only dephosphorylated Cx43 changed their localization to the plasma membrane, whereas phosphorylated Cx43 remained at the intercalated disk (Fig. 3a). This redistribution did not occur homogeneously in the heart, but appeared as a patchy distribution (Fig. 3b).

Next we performed a quantitative histological analysis of the phosphorylation status of Cx43 (Fig. 4). We measured the area of the cardiomyocyte which has the diffuse signal at the entire plasma membrane (dephosphorylated area). The dephosphorylated area at the end of perfusion was $5.3 \pm 1.1\%$ in non-hypoxia control heart and became greater as the hypoxic time increased ($48.2 \pm 3.4\%$ in 20 min-hypoxia and $67.9 \pm 2.3\%$ in 40 min-hypoxia group [$p < 0.01$]). Pretreatment of diltiazem significantly reduced the dephosphorylated area of Cx43 after hypoxia reoxygenation ($27.3 \pm 6.1\%$ in 20 min-hypoxia, and $48.3 \pm 4.3\%$ in 40 min-hypoxia group [$p < 0.01$ vs. non-hypoxia control]).

Western blot results supported the immunohisto-logical area analysis (Fig. 5). The higher band (44-46 kDa) indicates phosphorylated form and the lower band (41 kDa) the dephosphorylation form. In the control heart (0 min-hypoxia), the band density was predominantly in the higher band. However after hypoxic stress, the lower band (dpCx43) was stronger, with weakening of the higher band. Pretreatment with diltiazem (D+) attenuated the dephosphorylation change and preserved Cx43 phosphorylation status. Additionally, there was good correlation between dpCx43 and %RPP (Fig. 6, $y = -0.85x + 0.94$, $p < 0.01$).

Discussion

We have previously reported that Cx43 is highly phosphorylated in normal heart and dephosphorylated under hypoxic conditions, and that the amount of dephosphorylated Cx43 correlates inversely with the percent rate-pressure product (Matsushita et al., 2006). Diltiazem is a drug known as a cardiac protective agent to reduce arrhythmia on ischemic heart disease. However, it remains unclear how diltiazem plays a protective effect of cardiac function against ischemia. In this study, we assessed the effect of diltiazem on the heart function and the change of phosphorylation status of Cx43 during hypoxia-reoxygenation injury, and clarified the relationship between them by using immunohistochemical and immunoblot analyses in isolated rat heart.

When the heart is exposed to ischemia, intracellular calcium ($[Ca^{2+}]i$) gradually increases during 9-15 min after onset of ischemia (Steenbergen et al., 1987) and peaks immediately after reperfusion (Marban et al., 1990; Meissner and Morgan, 1995). This massive increase of $[Ca^{2+}]i$, so-called calcium overload, can be large enough to destroy cardiomyocytes, potentially resulting in arrhythmia by interruption of conduction propagation through the myocardium (Opie and Coetzee, 1988; Kihara and Morgan, 1991). The main pharmacological effect of diltiazem is blockage of the transmembrane calcium influx. Diltiazem thus protects the cardiomyocytes against calcium overload. Furthermore, it also slows conduction velocity, resulting in the delay of atrio-ventricular conduction and a decrease in the heart rate (Schwartz et al., 1985). Other important roles of diltiazem have been reported such as preservation of high-energy phosphates (i.e. adenosine triphosphate (ATP) and creatine phosphate), and a reduction of oxygen demand during ischemia (Weishaar et al., 1979; Sato et al., 1999; Takeo et al., 2004). In this experiment, HR was slowed by diltiazem pre-treatment, although it recovered to baseline at the end of perfusion in all groups. In contrast, contractile force reflected as LVDP was not significantly depressed by diltiazem, and it was improved at the end of reoxygenation in diltiazem treated groups compared to untreated ones. Hence %RPP, the oxygen consumption index (multiply HR by LVDP), was higher during the reoxygenation period in D+ compared to D-, although %RPP was depressed around 70% of baseline by treatment of diltiazem. In addition, left ventricular end-diastolic pressure (LVEDP) was constantly lower in D+ through both hypoxia and reoxygenation period. GOT release was reduced immediately after reoxygenation in D+, most likely due to diltiazem preventing rigor contraction of cardiomyocytes caused by calcium overload. These data indicated that diltiazem preserved oxygen consumption and protected the heart from cell destruction. The net effect was preservation of whole LV function by protection of cardiomyocytes against hypoxia-reoxygenation injury. Other inotropic medicines known to reduce the ATP consumption such as beta-blocker or Ca-channel blocker, are expected to play the similar role as Diltiazem does to cardiomyocytes.

Our group and others have previously shown that most Cx43 in normal heart is phosphorylated at many sites, including serine 368 (Ser368), and is located at the intercalated disk to provide intercellular communication via gap junction in vivo and in vitro (Musil et al., 1991; Beardslee et al., 2000). However when the heart was subjected to hypoxia, Cx43 was dephosphorylated and changed its distribution from intercalated disks to the entire plasma membrane (Beardslee et al., 2000; Turner et al., 2004, Matsushita et al., 2006). The mechanism of this change in phosphorylation status and localization of Cx43 is not yet fully elucidated, but is likely related to ischemia, hypoxia, low pH or $[Ca^{2+}]_i$ (Burt, 1997; Beardslee et al., 2000; Turner et al., 2004; Matsushita et

al., 2006). It has also been reported that the depression of intracellular ATP concentration promotes dephosphorylation of Cx43 (Beardslee et al., 2000). In this study we showed that there is less Cx43 dephosphorylation in the diltiazem pre-treated group, suggesting that the preservation of intracellular ATP by diltiazem also preserves the phosphorylation of Cx43.

When Cx43 was dephosphorylated, the protein migrated from the intercalated disk (ID) to the entire plasma membrane. It has been suggested that this redistribution may be caused by dissociation of Cx43 and ZO-1, a tight junction protein which is located at the ID (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998), and that only phosphorylated Cx43 can bind to ZO-1 (Toyofuku et al., 2001). Interestingly, as shown in Fig. 3a, dephosphorylated Cx43 was located both IDs and the entire plasma membrane after hypoxia, although all of the phosphorylated Cx43 still remained at the IDs. One interpretation is that Cx43 molecules are dephosphorylated at ID and thereby disconnected from ZO-1, resulting in unanchored Cx43 that is freed to move laterally onto the plasma membrane. Due to this redistribution, the phosphorylation change of Cx43 protein was quantified by using the immunohisto-chemical approach. The three kinds of serine phosphorylation specific antibodies (Ser368) were used to reconfirm the phosphorylation status of Cx43 in this study. The signal distribution and phosphorylation change among the three antibodies were consistent in both immunohistochemistry and immunoblotting results (Matsushita et al., 2006). Immunohistochemstry results showed that dephosphorylated Cx43 was not distributed uniformly, but rather as patch-like islands. This redistribution likely causes the blockage of the electrical signal, and conductive propagation takes an alternative route, creating the substrate for reentrant circuits (Smith et al., 1991; Peters et al., 1997). Cx43 protein was partly returned back to the phosphorylated form by reoxygenation, and its distribution partly returned to the intercalated disk pattern (Ai and Pogwizd, 2005; Matsushita et al., 2006). The retained amount of dephosphorylated Cx43 at the end of reoxygenation depended on the length of the hypoxia exposure time (Matsushita et al., 2006). In addition, the "dephosphorylated" area was negatively-correlated with %RPP as in our previous report (Matsushita et al., 2006). In this study, pretreatment with diltiazem prevented the dephosphorylation of Cx43 and also preserved %RPP. There was still negative-correlation between them under both non-treated and treated conditions. Hence, it is suggested that detecting the amount of dephosphorylated Cx43 can be useful tool for assessing the extent of heart damage.

In conclusion, we demonstrated that pretreatment with diltiazem preserves cardiac function by protecting against hypoxia-reoxygenation injury, and preserves the phosphorylation status of Cx43 at Ser368. The preservation effect of Cx43 phosphorylation may decrease the risk of reentrant arrhythmia by inhibiting the disturbance of intercellular communication.

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