Down-regulation of Connexin43 in Early Myocardial Ischemia and Protective Effect by Ischemic Preconditioning in Rat Hearts *In Vivo*

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SUMMARY

Connexin 43 (Cx43), a primary component of gap junctions, contributes to intercellular electrochemical communication. Cx43 undergoes dephosphorylation in early ischemia. We examined whether Cx43 is degraded in association with dephosphorylation during early myocardial ischemia and whether ischemic preconditioning (IP) affects the degradation after rat coronary artery occlusion. Male Sprague-Dawley rats underwent coronary artery occlusion for 1, 2, or 3 hours, or for 1 hour following treatment either with a calcineurin inhibitor (cyclosporine A), proteasome inhibitor (PSI), or lysosomal inhibitor (E64c), or following IP alone or after protein kinase C (PKC) inhibitor (chelerythrine) pretreatment. The IP was afforded by three cycles of 3 minute ischemia and 5 minute reperfusion. A large portion of the phosphorylated Cx43 (pCx43) in the membrane fraction was dephosphorylated, while a small portion was degraded at 1 hour of ischemia. The effects of the inhibitors were dephosphorylation and degradation by calcineurin and proteasome/lysosome, respectively. IP suppressed the decrease in pCx43 and increase in dCx43, while only the former was inhibited by the PKC inhibitor chelerythrine. The Cx43 mRNA level was reduced at 3 hours, but not at 1 hour of ischemia, irrespective of IP. We believe that Cx43 is dephosphorylated and degraded in early ischemia, whereas Cx43 transcription was suppressed at a later phase of ischemia. (Jpn Heart J 2004; 45: 1007-1019)

Key words: Connexin 43, Myocardial ischemia, Dephosphorylation, Degradation, Ischemic preconditioning, Protein kinase C

CARDIAC gap junctions (GJ), composed mainly of connexin43 (Cx43), regulate electrochemical coupling between cardiomyocytes, thereby coordinating the contraction.¹⁾ Gap junctional properties can be regulated by protein kinase C (PKC), mitogen-activated protein kinase, and src protein kinase, among others.¹⁾

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Cx43 immunoreactivity lies primarily in the intercalated discs (ID) and its intensity is reduced during myocardial ischemia,^{2,3)} which may implicate it arrhythmogenesis.⁴⁾ In Cx43-deficient mice, ischemia promotes ventricular arrhythmias.⁵⁾ It has been also demonstrated that dephosphorylation and translocation of Cx43 from the GJ is associated with electrical uncoupling induced by ischemia.⁶⁾ On the basis of the findings that hypoxia induces Cx43 dephosphorylation through calcineurin and reduces Cx43 immunostaining in the astrocytes, it has been proposed that “epitope masking” due to dephosphorylation reduces immunostaining.⁷⁾ This hypothesis has not been evaluated in myocardial ischemia.

In normoxic perfused hearts and cultured cardiomyocytes, Cx43 turns over rapidly with a half-life of about 1.3 hours through proteasome and/or lysosomal proteases.^{1,8)} This rapid Cx43 turnover is expected to regulate GJ assembly and conductance under physiological and pathological conditions.¹⁾ However, little is known about the role of these proteases in Cx43 turnover in *in vivo* myocardial ischemia.

Ischemic preconditioning (IP), conferred by brief ischemia-reperfusion cycles, attenuates arrhythmias, myocardial injury, left ventricular remodeling, and infarct development after subsequent prolonged ischemia (-reperfusion).⁹⁻¹¹⁾ It is known that PKC and ATP-sensitive K⁺ channels are involved in the protection afforded by IP.^{10,12)} It is reported that Cx43 is involved in the infarct size limitation afforded by IP as demonstrated by the comparison of heterozygous knockout and wild type mice.¹³⁾ Recently, it has been proposed that the protective effect of IP against myocardial infarction is exerted at the GJ through Cx43 modulation in ischemic myocardium.¹⁴⁻¹⁶⁾

In an *in vivo* model of early myocardial ischemia in rats, we addressed 1) whether Cx43 is degraded in association with dephosphorylation, or reduced immunostaining, 2) whether IP affects these changes, and 3) whether the transcription is modulated.

METHODS

Experimental animals and protocols: In accordance with the Guidelines of the Committee on Animal Experiments of the Graduate School of Medicine, University of Tokyo, male Sprague-Dawley rats, aged 8 weeks, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and ventilated with room air with the body temperature maintained at 37-38°C, as previously reported.^{3,10)} After left thoracotomy, the left anterior descending coronary artery (LAD) was occluded. The rats were randomly assigned to one of the following groups (*n* = 5 for each). In the time course study, the rats underwent LAD occlusion for 1, 2, or 3 hours. In the

inhibitor study, the rats were pretreated either with the calcineurin inhibitor cyclosporine A (CsA) (Wako Pure Chemicals, Osaka, Japan) (5 mg/kg, i.v.) for 15 minutes, the lysosomal inhibitor E64c (Peptide Institute, Osaka, Japan) (10 mg/kg, i.p.) for 1 hour, or the proteasome inhibitor PSI (Peptide Institute) (2 mg/kg, i.p.) for 1 hour before LAD occlusion. In the IP study, the rats were subjected to an IP procedure consisting of 3 cycles of 3 minutes of ischemia and 5 minutes of reperfusion¹⁰⁾ before ischemia for 1 or 3 hours. To another group, the PKC inhibitor chelerythrine chloride (Sigma, St. Louis, MO, USA) (5 mg/kg, i.v.) was administered immediately before the IP procedure. CsA, E64c, PSI, and chelerythrine were dissolved in dimethyl-sulfoxide (DMSO) and diluted with physiological saline at final DMSO concentrations of 2% v/v before injection. We determined the amount and the route of the inhibitors on the basis of previous results.^{10,17-19)} The sham group rats underwent the same procedure without LAD occlusion, while the experimental periods and the vehicle volume were matched. At the end of the LAD occlusion, following the injection of 0.3% Evans blue solution, the hearts were quickly removed and rinsed with physiological saline. The ischemic area, demarcated from the dye-stained nonischemic area,¹⁰⁾ was carefully dissected and frozen at -80°C until the analyses.

Biochemical analyses: As previously described,²⁰⁾ the frozen hearts underwent subcellular fractionation. The 1,000 × g pellet, 100,000 × g pellet, and 100,000 × g supernatant were referred to as the P1 (nuclear-myofibril), P2 (membrane), and S (cytosolic) fractions, respectively.^{20,21)} SDS-polyacrylamide gel (12.5%) electrophoresis and immunoblotting were performed as previously described,⁶⁾ with equal protein (10 µg/lane) loading. The PVDF membranes were incubated with the anti-Cx43 antibody (mouse monoclonal antibody or rabbit polyclonal antibody, Zymed Laboratories, South San Francisco, CA, USA) for 1 hour, and then the proteins on the membranes were visualized using an ECL Western blotting detection kit (Amersham Life Science, Buckinghamshire, UK). The band densities were quantified using an image analyzer (Densitograph AE-6920, ATTO, Tokyo).¹⁰⁾ For dot blotting, 25 µg aliquots of the S fraction were spotted onto nitrocellulose membranes, which were dried and processed in a manner similar to that for the immunoblotting procedure.

Immunohistochemistry: Immunofluorescence staining was performed as previously described.^{3,6)} Paraformaldehyde-fixed and paraffin-embedded heart sections were treated with microwaves, blocked with 5% BSA/TBS, incubated with the anti-Cx43 antibody overnight at 4°C, washed with TBS, and incubated with fluorescein isothiocyanate-labeled IgG and Texas-Red (r)-labeled IgG (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. The tissue sections were examined under immunofluorescence microscopy (Axioskop 2,

Zeiss, Jena, Germany) equipped with an image analyzer (Image-Pro Plus, Media Cybernetics, Maryland, USA).

Northern blotting analysis: The Northern blotting was performed as previously described.²²⁾ Total RNA (2 μ g), extracted from the frozen heart, was reverse-transcribed to cDNA and amplified by polymerase chain reaction (PCR) with sequence specific primers: Cx43 sense 5'-TGGTACATCTATGGGTTCAGCT-TGAGCGCG-3', and antisense 5'-CATAATCGACAGCTCGATGCTCAAGC-CGGT-3'. The PCR product was recovered as a probe and labeled using a digoxigenin-11-deoxyuridine-5-triphosphatase (DIG) labeling kit (Boehringer Mannheim, Mannheim, Germany). Total RNA (20 μ g) was denatured, electrophoresed on 1% agarose gels, and transferred onto nylon membranes. After hybridization, the band was detected with a DIG detection kit (Boehringer Mannheim), and the density was quantified using the image analyzer and normalized to the 28S rRNA.

Statistics: The data are expressed as the mean $\pm$ SE and were analyzed statistically by analysis of variance (ANOVA) followed by Fisher's post hoc analysis. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Temporal changes in Cx43 levels during early myocardial ischemia: The anti-Cx43 polyclonal antibody (pAb) reacts both with phosphorylated Cx43 (pCx43) and dephosphorylated Cx43 (dCx43), whereas the anti-Cx43 monoclonal antibody (mAb) reacts selectively with dCx43.²³⁾ Figure 1 shows representative immunoblots of the P2 fraction, while Figure 2 shows the quantification of the

Figure 1. Representative immunoblots showing the temporal change in the Cx43 levels of the P2 fraction at 0, 1, 2, and 3 hours of ischemia (43 kDa = phosphorylated Cx43, 41 kDa = dephosphorylated Cx43). The anti-Cx43 polyclonal antibody recognizes both phosphorylated Cx43 and dephosphorylated Cx43 bands (upper blot), whereas the anti-Cx43 monoclonal antibody only recognizes dephosphorylated Cx43 bands (lower blot).

Figure 2. The relative densities of the Cx43 bands on the immunoblots are quantified as ratios relative to the mean densities before (0 hour) ischemia (**A**: phosphorylated Cx43, **B**: dephosphorylated Cx43, **C**: total Cx43). The significant differences between values at each 1 hour interval ($*P < 0.05$) are shown. Figure 2D represents the relative densities of Cx43 spots of the S fraction on dot blot quantified as ratios relative to the mean density before (0 hour) ischemia. The significant differences between values at each 1 hour interval ($*P < 0.05$) are shown.

Cx43 band density on the immunoblots (A, B, C) and the Cx43 dot density of the S fraction on the dot blots (D). The P2 contains the ID and sarcolemma, while the S contains the cytosol.²⁴⁾ At 1 hour of ischemia, the pCx43 in the P2 fraction was reduced to about 30% of the levels of the sham group, whereas the dCx43 levels greatly increased, indicating the dephosphorylation of pCx43. Total Cx43 (pCx43 plus dCx43) was decreased by only about 15% at 1 hour of ischemia. The pCx43 levels were greatly decreased by 3 hours of ischemia, along with the reduction of dCx43 between 1 and 3 hours of ischemia. The Cx43 levels in the nonischemic area were unaltered by 3 hours of ischemia, while a Cx43 band was not detected

in the P1 and S fractions (data not shown). Dot-blotting analysis of the S fraction showed an increase in Cx43 immunoreactivity with ischemia duration.

There was neither a reduction in triphenyltetrazolium chloride staining (infarction) nor myoglobin diffusion after 3 hours of ischemia (data not shown). By contrast, infarct size was about 24% after 3 days of ischemia, and the infarct and myoglobin diffusion were evident after approximately 30 minutes of ischemia followed by reperfusion for more than about 3 hours in the same models (data not shown).

Figure 3. Immunofluorescence localization of Cx43 with the anti-Cx43 polyclonal antibody (**A, D, G**) and monoclonal antibody (**B, E, H**) on sections from the sham (**A, B, C**), 1 hour of ischemia (**D, E, F**), 3 hours of ischemia (**G, H, I**), and the corresponding fused images (**C, F, I**). The polyclonal antibody stains the intercalated discs of the sham, whereas it stains the cytoplasm diffusely but weakly at 1 and 3 hours of ischemia. The monoclonal antibody stains the intercalated discs at 1 hour (strongly) and 3 hours of ischemia. (magnifications: $\times 400$).

Figure 3 shows the immunofluorescence profiles of Cx43 in ischemic heart. In the sham, the total Cx43 levels, as detected by the pAb, were distributed to the ID (A, B, C). At 1 hour of ischemia, the dCx43 signal, as detected by mAb, was greatly increased in the ID (E, F), with a small decrease in the pCx43 signal (D). In the later phase of ischemia (1-3 hours), the total Cx43 signal in the ID was clearly reduced and diffusely distributed throughout the cytoplasm (G, H, I). Thus, pCx43 in the ID was largely dephosphorylated during early myocardial ischemia, whereas total Cx43 was reduced during the later phase of ischemia, which is consistent with the biochemical analyses (Figure 2C).

Effect of cyclosporine A on Cx43 levels: We examined the effects of a calcineurin inhibitor, CsA, on the Cx43 in immunoblots after ischemia for 1 hour. CsA inhibited dephosphorylation of Cx43 at 1 hour of ischemia with no effect on the total Cx43 level (Figure 4).

Effects of proteasome and lysosome inhibitors on Cx43 levels: We investigated the effects of a proteasome inhibitor, PSI (Figure 5A), and a lysosomal inhibitor,

Figure 4. Representative immunoblots with the anti-Cx43 polyclonal antibody (upper) and monoclonal antibody (lower) showing the effect of CsA on phosphorylated Cx43 and dephosphorylated Cx43 levels at 1 hour of ischemia and in the sham. The relative densities of phosphorylated Cx43 and dephosphorylated Cx43 are quantified as ratios relative to the mean densities of the sham without CsA (* $P < 0.05$ versus ischemia without CsA).

Figure 5. Representative immunoblots with the anti-Cx43 polyclonal antibody (upper) and monoclonal antibody (lower) showing the effects of PSI (A) and E64c (B) on phosphorylated Cx43 and dephosphorylated Cx43 levels at 1 hour of ischemia and in the sham. The relative densities of phosphorylated Cx43 and dephosphorylated Cx43 are quantified as ratios relative to the mean densities of the sham without inhibitors (* $P < 0.05$ versus ischemia without inhibitor).

Figure 6. Representative immunoblots with the anti-Cx43 polyclonal antibody (upper) and the monoclonal antibody (lower) showing the effects of ischemic preconditioning (IP) (A) and the PKC inhibitor chelerythrine (Che) (B) on phosphorylated Cx43 and dephosphorylated Cx43 levels at 1 hour of ischemia. The relative densities of phosphorylated Cx43 and dephosphorylated Cx43 are quantified as ratios relative to the mean densities of the sham (* $P < 0.05$ versus ischemia without IP, † $P < 0.05$ versus IP without chelerythrine).

Figure 7. Representative Northern blot showing Cx43 mRNA after 1 and 3 hours of ischemia/ $\pm$ IP. The density of each Cx43 band was normalized with reference to that of the 28S rRNA band for each sample and expressed as ratios relative to the mean density of the sham (* P < 0.05 versus sham).

E64c (Figure 5B), on Cx43 levels after ischemia for 1 hour. PSI and E64c both inhibited the pCx43 decrease to a similar extent, with no effect on dCx43. In the nonischemic heart, the inhibitors per se did not increase the Cx43 band intensity.

Effects of ischemic preconditioning and PKC inhibition on Cx43 levels: As shown in Figure 6A, IP inhibited the decrease in pCx43 and increase in dCx43 at 1 hour, but not at 3 hours of ischemia. However, a selective PKC inhibitor chelerythrine inhibited the effect of IP on the pCx43 level but not that on dCx43 level at 1 hour of ischemia, as demonstrated in Figure 6B. Chelerythrine had no effect on Cx43 levels in normoxic heart. We used chelerythrine because of its selectivity and effectiveness from our previous studies in perfusion and permanent occlusion models in the rat heart.^{10,26)}

Cx43 mRNA levels: As shown in Figure 7, Cx43 mRNA was decreased at 3 hours but not at 1 hour of ischemia, irrespective of IP, suggesting transcriptional down-regulation in the later phase of ischemia.

DISCUSSION

We demonstrated for the first time that proteolysis of Cx43 is enhanced, while transcription is suppressed during early myocardial ischemia in an *in vivo*

permanent occlusion model of the rat. At 1 hour of ischemia, dCx43 was largely increased, which was inhibited by a calcineurin inhibitor. pCx43 was decreased by 15%, which was inhibited either by a lysosomal inhibitor or proteasome inhibitor. IP inhibited these changes, though PKC inhibition suppressed only the change in pCx43. The down-regulation of Cx43 may cause reduced Cx43 immunostaining postischemia.⁷⁾

By 1 hour of ischemia, a large portion of pCx43 was dephosphorylated and a small portion of Cx43 was supposed to be degraded (Figures 1, 2), which is consistent with a previous study showing that Cx43 is dephosphorylated in the perfused heart rendered ischemic for 40 minutes.⁶⁾ This Cx43 dephosphorylation during 1 hour of ischemia, as shown by the conversion of pCx43 to dCx43, was clearly inhibited by CsA (Figure 4), indicating the involvement of calcineurin. However, CsA did not affect the Cx43 down-regulation at 1 hour of myocardial ischemia. Additionally, both pCx43 and dCx43 were reduced with the duration of ischemia from 1 to 3 hours, supporting degradation independent of dephosphorylation. On the basis of these findings, we believe that proteolysis rather than "epitope masking by dephosphorylation" causes the "reduced immunostaining for Cx43 in ischemic myocardium".^{2,3)} Additionally, these changes precede the development of myocardial injury as demonstrated by the negative myoglobin diffusion or reduced triphenyltetrazolium chloride staining (data not shown), in the same model in which these changes can be reproduced after ischemia-reperfusion or prolonged ischemia (data not shown).

From the results of inhibitor studies in normoxic perfused hearts and cardiomyocytes, it has been formerly proposed that lysosomal proteases preferentially degrade pCx43 and other internalized membrane proteins after endocytosis, whereas proteasomes degrade dCx43, miss-folded or oxidized proteins during transport through the endoplasmic reticulum/Golgi apparatus in cardiomyocytes.^{1,8)} However, we found that a lysosomal inhibitor and a proteasomal inhibitor suppressed the reduction in the pCx43 at 1 hour of ischemia, though to a much smaller extent than the increase in dCx43 (Figure 5). These findings also appear to contradict the causality between dephosphorylation and proteolysis as well as the preference of the two protease systems in *in vivo* myocardial ischemia. The finding that E64c and PSI inhibited pCx43 reduction to similar extents suggests that both lysosomes and proteasomes are sequentially or simultaneously involved in Cx43 degradation in myocardial ischemia.

Immunohistochemical study showed that dCx43 label (by pAb) in the ID became distinct at 1 hour of ischemia, whereas pCx43 and dCx43 labels (by mAb) became diffusely distributed to the intracellular spaces thereafter (Figure 3). Since lysosomal and proteasomal proteases are distributed to the intracellular spaces but not to the ID, these findings suggest that Cx43 undergoes proteolysis

in situ beneath the ID or after endocytosis. The protease inhibitors were ineffective in the reductions in pCx43 and dCx43 at 3 hours of ischemia. This is not surprising, as no more inhibitor can reach the ischemic area after permanent coronary occlusion. On the other hand, consistent with the enhanced immunolabeling with time of ischemia throughout the myocardium (Figure 3), the Cx43 immunoreactivity in the S fraction on dot blots was increased with ischemia duration (Figure 2D). These data suggest that, as a result of rapid and efficient degradation during myocardial ischemia, the Cx43 epitope accumulates in the cytosol. Alternatively, given that the anti-pCx43 antibody is raised against its C-terminal peptides, the immuno-labeling data could be explained if the C-terminal region of Cx43 facing the cytoplasm was to be partially cleaved during early myocardial ischemia. Collectively, our data have clarified for the first time that myocardial ischemia reduced Cx43 levels by enhanced proteolysis. In addition to the enhanced degradation, the Cx43 level is supposed to be reduced by transcriptional down-regulation at 3 hours of ischemia because the Cx43 mRNA level was greatly reduced at 3 hours of ischemia (Figure 7).

IP inhibited the decrease in pCx43 level and increase in dCx43 at 1 hour postischemia, but only the change in pCx43 was suppressed by the PKC inhibitor (Figure 6). This observation suggests that PKC mediates the effect of IP on proteolysis rather than that on dephosphorylation as reported in previous immunohistochemical studies.^{15,16)} However, we were unable to discriminate between the two mechanisms due to the limitations of study in an *in vivo* model. To address the latter issue, we have begun studies using molecular and cell biological approaches.

It is reported that IP attenuates cardiac arrhythmias and infarct development,^{9,10)} while previous studies on Cx43 knock-out mice support the link between Cx43 down-regulation and arrhythmias.⁵⁾ It was also shown that a gap junction blocker, heptanol, attenuates the protective effect of IP.²⁵⁾ These data suggest that IP afforded protection against myocardial ischemia through the propagation of survival signals through Cx43-based GJ. The pathophysiological implications need to be examined in further molecular and cell biological studies.

In conclusion, during early ischemia (~1 hour), Cx43 undergoes dephosphorylation and to a smaller extent proteolysis. During the later phase of ischemia (~3 hours), transcription is suppressed and proteolysis is enhanced.

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