Rapid Turnover of Connexin43 in the Adult Rat Heart

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Abstract—Remodeling of the distribution of gap junctions is an important feature of anatomic substrates of arrhythmias in patients with healed myocardial infarcts. Mechanisms underlying this process are poorly understood but probably involve changes in gap junction protein (connexin) synthesis, assembly into channels, and degradation. The half-life of the principal cardiac gap junction protein, connexin43 (Cx43), is only 1.5 to 2 hours in primary cultures of neonatal myocytes, but it is unknown whether rapid turnover of Cx43 occurs in the adult heart or is unique to disaggregated neonatal myocytes that are actively reestablishing connections in vitro. To characterize connexin turnover dynamics in the adult heart and to elucidate its potential role in remodeling of gap junctions, we measured Cx43 turnover kinetics and characterized the proteolytic pathways involved in Cx43 degradation in isolated perfused adult rat hearts. Hearts were labeled for 40 minutes with Krebs-Henseleit buffer containing [35S]methionine, and then chase perfusions were performed with nonradioactive buffer for 0, 60, 120, and 240 minutes. Quantitative immunoprecipitation assays of Cx43 radioactivity in 4 hearts at each time point yielded a monoexponential decay curve indicating a Cx43 half-life of 1.3 hours. Proteolytic pathways responsible for Cx43 degradation were elucidated by perfusing isolated rat hearts for 4 hours with specific inhibitors of either lysosomal or proteasomal proteolysis. Immunoblot analysis demonstrated significant increases (≈30%) in Cx43 content in hearts perfused with either lysosomal or proteasomal pathway inhibitors. Most of the Cx43 in hearts perfused with lysosomal inhibitors consisted of phosphorylated isoforms, whereas nonphosphorylated Cx43 accumulated selectively in hearts perfused with a specific proteasomal inhibitor. These results indicate that Cx43 turns over rapidly in the adult heart and is degraded by multiple proteolytic pathways. Regulation of Cx43 degradation could play an important role in gap junction remodeling in response to cardiac injury. (Circ Res. 1998;83:629-635.)

Key Words: gap junction ■ connexin43 ■ proteolysis ■ anatomic substrate of arrhythmia

Remodeling of gap junction distributions appears to be a central feature of anatomic substrates of reentrant ventricular arrhythmias in patients with healed myocardial infarcts. Mechanisms responsible for this remodeling process are poorly understood but probably involve dynamic changes in gap junction protein (connexin) synthesis, assembly into channels, and degradation. The turnover of gap junction proteins in primary cultures of neonatal rat ventricular myocytes is surprisingly rapid. The half-lives of the ventricular gap junction proteins connexin43 (Cx43) and connexin45 (Cx45) in cultured cardiac myocytes have been reported to be 1.5 to 2.0 hours and ~3.0 hours, respectively. However, it is not known whether rapid turnover of connexins occurs in the adult heart or is merely a feature of disaggregated neonatal myocytes that are actively reestablishing intercellular connections in vitro.

To elucidate potential mechanisms responsible for remodeling of gap junction distributions in diseased myocardium, we performed the present study to measure the turnover kinetics of the major cardiac gap junction protein, Cx43, in the adult rat heart and to characterize the proteolytic pathways involved in its degradation. We observed that Cx43 turns over in the adult rat heart with a half-life of <1.5 hours and is degraded by both proteasomal and lysosomal pathways. These results indicate that gap junctions are remarkably dynamic structures in which protein subunits of junctional channels are turning over several times a day. Although the biological significance of rapid turnover of Cx43 is unclear, demonstration of such rapid kinetics under physiological conditions suggests that regulation of connexin degradation could play a role in remodeling of gap junction distributions in response to pathophysiological conditions.

Materials and Methods

Animal Care
All experimental procedures were approved by the Animal Studies Committee at Washington University School of Medicine and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).

Antibodies
A rabbit antiserum directed against a synthetic peptide of amino acids 252 to 271 in Cx43 was used in all immunoprecipitation experiments. This antibody has been characterized in previous
studies and has been shown to be monospecific for Cx43. A second rabbit polyclonal anti-Cx43 antibody, generated from a (His)_7-Cx43CT fusion protein corresponding to amino acids 212 to 382 of Cx43, was used in all immunoblot experiments. The specificity of this antibody has also been characterized in previous studies. A commercially available rabbit polyclonal anti-Cx43 antibody (Zymed) was used in all immunofluorescence experiments. Polyclonal rabbit antibodies against cardiac actin (Boehringer-Mannheim) and c-jun (Santa Cruz) were used in control experiments.

**Protease Inhibitors**

Leupeptin was purchased from Boehringer-Mannheim. All other reagents were obtained from Sigma Chemical Co.

**Isolated Heart Perfusions**

Adult male Sprague-Dawley rats (Sasco, O’Fallon, Mo), 300 to 324 g, were anesthetized with intraperitoneal ketamine (10 g/100 mL) and xylazine (10 g/100 mL) at a dose of 0.1 mL/100 g body wt. Hearts were excised rapidly and immersed in ice-cold cardioplegic solution containing (mmol/L) NaCl 77, KCl 40, MgCl₂ 32, CaCl₂ 2.2, glucose 276, and NaHCO₃ 4.9. Hearts were then mounted on a Langendorff perfusion apparatus and perfused retrogradely via aortic cannulation. Perfusion pressure and heart rate were monitored continuously with a dual-channel chart recorder connected to a pressure transducer placed 2 cm above the heart. In preparation for all metabolic labeling studies or experiments involving proteolysis pathway inhibitors, hearts were first perfused during a 15-minute washout stabilization period in nonrecirculating mode with standard Krebs-Henseleit buffer (KHB) containing 11 mmol/L glucose and 0.4% diazyl BSA and bubbled with 95% O₂/5% CO₂. Throughout this and all subsequent perfusion intervals, aortic perfusion pressures were maintained at 50 to 80 mm Hg by adjustments in flow rates. Cx43 turnover rates were determined in hearts that were metabolically labeled. After the initial 15-minute perfusion period, hearts were perfused for 40 minutes in recirculation mode with 40 mL of the same buffer containing 1.5 mCi [35S]methionine (3000 mCi/mmol). This “pulse” perfusion was “chased” by perfusing hearts with standard KHB containing glucose, BSA, and unlabeled L-methionine (440 μmol/L) for 0, 60, 120, or 240 minutes in nonrecirculating mode. Four separate hearts were studied at each chase perfusion interval. At the conclusion of the chase perfusion period, the hearts were freeze-clamped, trimmed of great vessels, and weighed. The frozen tissues were pulverized with a mortar and pestle that had been cooled in liquid nitrogen and were stored at −70°C for subsequent immunoprecipitation assays.

The effects of proteolysis pathway inhibitors on Cx43 were determined in additional adult rat hearts. After the initial 15-minute perfusion interval, hearts were perfused for 4 hours with standard KHB containing glucose, BSA, physiological concentrations of essential amino acids, and the protease inhibitors leupeptin (25 μmol/L), NHECl (10 mmol/L), or N-acetyl-leucyl-leucyl-norleucine (ALLN) (10 μmol/L). All protease inhibitors were dissolved in DMSO immediately before use and added to the perfusion buffer (0.05% DMSO final concentration in KHB). Control hearts were perfused with buffer containing only 0.05% DMSO.

**Immunoprecipitation and Immunoblot Analysis**

Tissues were prepared according to the method of Yoshida et al. Pulverized frozen heart samples were suspended in 2 vol of buffer containing (mmol/L) NaCl 50, Tris-HCl (pH 7.4) 20, EGTA 1, NaN₃ 5, β-mercaptoethanol 10, and phenylmethylsulfonyl fluoride 2 plus 20 μmol/L leupeptin and 150 mmol/L pepstatin A and homogenized with a Tissue Terror homogenizer (4 bursts, 30 seconds each). Homogenates were diluted with 4 vol of the same buffer and centrifuged at 10 000g for 30 minutes. Supernatants were discarded, and the remaining pellets were resuspended by brief sonication in radioimmunoprecipitation assay buffer (1% Triton X-100, 2% SDS, 0.01% NaF, 0.01% Na₃VO₄, 0.01% pepstatin, and 0.01% 4-[2-aminoethyl]benzenesulfonyl fluoride (Pefabloc) in PBS) for immunoprecipitation experiments or in electrophoresis buffer (125 mmol/L Tris-HCl [pH 6.8], 1 mmol/L EGTA, 2% SDS, and 5% β-mercaptoethanol plus protease inhibitors as described above) for immunoblot experiments. Incorporation of radioactivity into total proteins was assessed by trichloroacetic acid precipitation of aliquots from individual homogenates followed by liquid scintillation spectrometry.

Samples in radioimmunoprecipitation assay buffer were analyzed in immunoprecipitation assays according to the method of Laing et al. Briefly, samples were boiled for 5 minutes and centrifuged at 14 000 g for 15 minutes. Cx43 or actin was precipitated from the supernatants by the addition of 50 μL protein A–Sepharose beads plus 5 μL polyclonal rabbit antibodies directed against Cx43 or cardiac actin. After the beads were washed and resuspended in electrophoresis buffer, samples were separated by SDS-PAGE and analyzed by fluorography for 7 days at −70°C.

Protein concentrations in samples in electrophoresis buffer were determined by the technique of Bradford. Aliquots containing 30 μg total protein were separated by SDS-PAGE on 12.5% polyacrylamide gels and transferred to Immobilon P membranes (Millipore). Membranes were blocked overnight in PBS containing 0.5% Triton X-100 and 2% gelatin and then incubated for 3 hours with polyclonal antibodies against (His)7-Cx43CT or c-jun. After being washed, blots were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG, developed with ECL chemiluminescence reagent (Amersham), and exposed to medical x-ray film (Fuji).

**Alkaline Phosphatase Digestion**

Equal aliquots of heart homogenates were incubated for 16 hours at 4°C with fetal calf intestinal alkaline phosphatase (10 U/30 μg of protein sample, Boehringer-Mannheim) in alkaline phosphate buffer (Boehringer-Mannheim) or in buffer alone before being separated on SDS-PAGE gels and analyzed by immunoblotting as described above.

**Densitometric and Kinetic Analysis**

The intensities of bands on polyacrylamide gels were quantified by densitometry. Bands were imaged with a Nikon Scantouch scanner, and images were digitized with Adobe Photoshop 4.0 software. The relative amounts of Cx43 were calculated by subtracting the background gray scale value in each experiment. Gray scale values in each pulse-chase experiment were normalized to the 0-minute chase interval, which was assigned a value of 1.0. The first-order decay constant (k) was calculated from the first-order decay curves of the form y = e−kt, generated by the program SigmaPlot (Jandel Scientific). Half-life was determined using the formula t½ = 0.693/k.

**Immunohistochemistry and Confocal Microscopy**

Transmural blocks of left ventricular myocardium from selected hearts perfused with protease inhibitors were fixed in 10% formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and mounted on gelatin-coated slides. The sections were deparaffinized, placed in citrate buffer, and boiled in a microwave oven for 10 minutes to enhance specific immunostaining. The sections were incubated overnight with anti-Cx43 antibodies (Zymed) diluted 1:400 and then incubated with CY3-conjugated goat anti-rabbit IgG diluted 1:800 before being examined by laser scanning confocal microscopy. Multiple fields from each ventricular sample were digitized, and the proportion of total myocardial tissue area occupied by specific Cx43 immunofluorescent signal was quantified as previously described.

**Statistical Analysis**

Differences between groups treated with various proteolytic inhibitors were analyzed by using ANOVA with the Tukey post hoc test. A value of P<.05 was considered statistically significant. All data are expressed as the mean±SD except where indicated.

**Results**

Cx43 in Isolated Perfused Rat Hearts

To characterize Cx43 turnover dynamics in the adult heart, we developed a Langendorff perfusion approach for metabol-
ic labeling of newly synthesized proteins in pulse-chase experiments and elucidation of proteolysis pathways involved in Cx43 degradation. We found that hearts could be routinely perfused for 4 hours without significant changes in spontaneous heart rate or apparent contractile function. Immuno- blot analysis of Cx43 content was indistinguishable in lysates prepared from hearts that had been perfused with buffer for 4 hours and lysates prepared from freshly excised (nonperfused) hearts (Figure 1A; compare lanes 1 and 2). In either case, Cx43 was detected as 2 electrophoretically separable forms. Closely spaced polypeptides migrating between 43 and 46 kDa accounted for 81±62% (n=4) of the densitometric signal, and a band migrating at 41 kDa constituted the remaining 19±62% of the specific Cx43 signal. Incubation of the lysates with alkaline phosphatase resulted in the appearance of a single 41-kDa polypeptide (Figure 1A, lane 3). These results indicate that the more slowly migrating bands represented phosphorylated isoforms of Cx43, referred to by Musil and Goodenough17 as Cx43-P1 (43 kDa) and Cx43-P2 (46 kDa), and the 41-kDa band was nonphosphorylated Cx43 (Cx43-NP). Two major bands migrating at ~43 to 46 kDa were observed when hearts were perfused with buffer containing [35S]methionine, and Cx43 was isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography (Figure 1B). These bands presumably represented phosphorylated isoforms of Cx43 (P1 and P2 forms).

**Cx43 Turnover in Isolated Perfused Adult Rat Hearts**

To determine the half-life of Cx43 in the adult rat heart, we performed pulse-chase experiments. Isolated perfused hearts were metabolically labeled by perfusion for 40 minutes with buffer containing [35S]methionine, followed by chase perfusions for 0, 1, 2, or 4 hours with buffer containing unlabeled l-methionine. Cx43 was immunoprecipitated from 4 hearts at each chase interval, and the amount of radioactivity in Cx43 was analyzed by SDS-PAGE and fluorography. Radioactive Cx43 migrating as a doublet at 43 to 46 kDa disappeared over the course of the 4-hour chase period (Figure 2). Densitometric quantification in 4 separate experiments produced the composite decay curve shown in Figure 2. Analysis of this composite curve revealed that the data were best fit by a monoeponential function with a decay constant (k) of 0.0093, derived from the equation A_t = A_0 e^{-kt}, where A_t is the signal for the protein at time t, and A_0 is the initial protein signal. Analysis of the 4 separate decay curves resulted in a mean decay constant (k) of 0.0100 ± 0.0038 and a calculated half-life of 1.3 hours. The coefficient of determination for these data was r²=0.91. Attempts to fit the data to a biexponential function assuming 2 different peptide species with dissimilar half-lives resulted in 2 virtually identical decay curves.
decay constants \( k_1 = 0.0091 \), \( k_2 = 0.0089 \); \( r^2 = 0.94 \). These results suggest that a single pool of Cx43 decayed with first-order kinetics. 15

A control experiment was performed in which radioactive actin was quantified in immunoprecipitation assays after a 30-minute interval of metabolic labeling and 120 or 240 minutes of chase perfusions with unlabeled buffer as described above in Cx43 turnover experiments. As shown in Figure 2, actin was immunoprecipitated as an \( \sim 43\)-kDa band. There was significant labeling of actin after 30 minutes of perfusion with buffer containing radiolabeled methionine, but the amount of radioactivity in actin remained essentially unchanged after 2 or 4 hours of chase perfusion with unlabeled buffer.

Cx43 Content in Rat Hearts Perfused With Protease Inhibitors

To delineate the role of lysosomal and proteasomal proteolysis in the degradation of Cx43, isolated hearts were perfused for 4 hours with buffer containing glucose, BSA, physiological concentrations of essential amino acids, and specific inhibitors of the lysosomal and proteasomal pathways. Lysosomal inhibitors included leupeptin and the weak base NH\(_4\)Cl. Proteasomal proteolysis was inhibited by ALLN, which, at a concentration of 10 \( \mu \)mol/L, is highly specific for the proteasomal pathway. 18 During the 4-hour perfusions with each inhibitor, spontaneous heart rates and apparent contractile function were maintained. At the completion of the 4-hour perfusion interval, hearts were either snap-frozen for subsequent immunoblot analysis of Cx43 content or fixed in formalin for immunohistochemistry and analysis by confocal microscopy.

The specificity of proteasomal inhibition by ALLN was confirmed in a preliminary study in which the amount of c-jun, a protein that is specifically degraded by the proteasomal pathway,19 was measured in hearts after 4-hour perfusions with either NH\(_4\)Cl or ALLN. Densitometric analysis of immunoblots revealed that the amount of c-jun was not increased over control levels after perfusion with NH\(_4\)Cl but that c-jun levels were increased by 60% after perfusion with ALLN (data not shown).

As shown in Figure 3, inhibitors of both the lysosomal and the proteasomal pathways increased the total amount of Cx43 detected by immunoblot analysis. Densitometric quantification of 4 separate immunoblots for each inhibitor showed that the lysosomal inhibitors NH\(_4\)Cl and leupeptin increased the amount of Cx43 by 41\( \pm \)15% \( (P<0.05) \) and 27\( \pm \)9% \( (P<0.05) \), respectively (Figure 4, top). Treatment of hearts with the proteasomal inhibitor ALLN increased the amount of Cx43 by 33\( \pm \)13% \( (P<0.05) \) (Figure 4, top). It is apparent from examination of the Cx43 band patterns in Figure 3 that perfusion of hearts with lysosomal inhibitors led to selective accumulation of phosphorylated isoforms of Cx43, whereas treatment with the proteasomal inhibitor caused an increase in
the nonphosphorylated isoforms. Densitometric quantification of the individual band intensities revealed that virtually all of the increase in total cardiac Cx43 signal was due to an increase in phosphorylated isoforms in hearts treated with lysosomal inhibitors (Figure 4, middle) and an increase in nonphosphorylated Cx43 in hearts treated with the proteasomal inhibitor ALLN (Figure 4, bottom). The lysosomal pathway inhibitors leupeptin and NH₄Cl did not differ in their effects on accumulation of Cx43-P1 versus Cx43-P2 isoforms. Thus, signals from these bands were combined in Figure 4 to indicate total phosphorylated Cx43.

To determine the effects of protease inhibitors on gap junction structures, we performed quantitative confocal microscopic analysis of ventricular tissues stained with anti-Cx43 antibodies. As shown in Figure 5, immunoreactive signal was concentrated at points of intercellular apposition in a pattern consistent with the known distribution of gap junction structures, we performed quantitative confocal microscopic analysis of ventricular tissues stained with anti-Cx43 antibodies. As shown in Figure 5, immunoreactive signal was concentrated at points of intercellular apposition in a pattern consistent with the known distribution of gap junction structures. There was a noticeable increase in the amount of Cx43 signal at points of intercellular apposition in sections of hearts treated for 4 hours with lysosomal and proteasomal inhibitors. Digital image processing revealed that the proportion of total myocardial tissue occupied by strong Cx43 immunoreactive signal in-...
Cx43 immunofluorescent signal localizes to sites of intercalated disks. Previous studies by Musil and Goodenough have indicated that phosphorylation of Cx43 is associated with its assembly into gap junctions. Phosphorylation may also target the protein for subsequent degradation. Although both lysosomal and proteasomal inhibitors increased Cx43 immunohistochemical signal at sites of apparent intercellular apposition, as judged by confocal microscopy, inhibition of lysosomal proteolysis led to selective accumulation of phosphorylated Cx43, whereas proteasomal inhibition caused nonphosphorylated Cx43 to accumulate. These results indicate that lysosomal and proteasomal inhibitors cause different populations of Cx43 to accumulate at or near the intercalated disk.

In general, degradation of internalized extracellular proteins and some membrane proteins occurs via the lysosomal pathway. Inhibition of lysosomal degradation might, therefore, prevent endocytotic removal of phosphorylated Cx43 from gap junctions, which could lead to selective accumulation of phosphorylated Cx43 and increased immunoreactivity at the cell surface. Proteasomal proteolysis is thought to be the major pathway for degradation of cytosolic and nuclear proteins and digestion of misfolded proteins trafficking through the endoplasmic reticulum and Golgi apparatus. Thus, ALLN might inhibit a proofreading step responsible for removing misfolded or unoligomerized Cx43 in the endoplasmic reticulum/Golgi, which would account for some selective accumulation of nonphosphorylated Cx43. This would not explain, however, why inhibition of proteasomal proteolysis by ALLN resulted in accumulation of Cx43 immunoreactivity at the cell surface. Taken together with the results of Musil and Goodenough, our results are consistent with the hypothesis that Cx43 phosphorylation is necessary for channel assembly and insertion into gap junctions but Cx43 can undergo dephosphorylation while still residing at the cell surface. An alternate interpretation of our data is that Cx43 phosphorylation is not a prerequisite for channel assembly and insertion into myocardial gap junctions. Further studies will be required to elucidate the role of connexin phosphorylation/dephosphorylation in gap junction channel assembly, function, and turnover.

Reentrant arrhythmias in patients with healed myocardial infarcts depend on regions of slow heterogeneous conduction and unidirectional conduction block that typically map to viable but structurally altered myocardium bordering the healed infarct scar. Conduction abnormalities cannot be explained by marked alterations in active sarcolemmal currents. However, morphometric and immunofluorescence studies have revealed extensive remodeling of the spatial distribution of gap junctions in myocardium bordering infarct scars. Thus, alterations in intercellular current transfer at gap junctions appear to play a critical role in the development of anatomic substrates of arrhythmias in patients with ischemic heart disease. During the active phase of infarct healing, viable myocytes at the edge of the infarct lose the normal pattern of large gap junctions concentrated at the ends of myocytes and exhibit a distinctly different pattern in which many small junctions become distributed uniformly over the cell surface. The potential role of changes in cardiac connexin degradation in this remodeling process is unknown. Our results suggest, however, that under physiological conditions, gap junctions are highly dynamic structures, and regulation of connexin degradation pathways could be an important mechanism in remodeling in response to pathophysiologic stimuli. It may also be possible to intervene with specific drugs targeted to connexin degradation pathways to limit anatomic changes that increase the risk of developing reentrant arrhythmias.

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