Dephosphorylation and Intracellular Redistribution of Ventricular Connexin43 During Electrical Uncoupling Induced by Ischemia


Abstract—Electrical uncoupling at gap junctions during acute myocardial ischemia contributes to conduction abnormalities and reentrant arrhythmias. Increased levels of intracellular Ca\(^{2+}\) and H\(^+\) and accumulation of amphipathic lipid metabolites during ischemia promote uncoupling, but other mechanisms may play a role. We tested the hypothesis that uncoupling induced by acute ischemia is associated with changes in phosphorylation of the major cardiac gap junction protein, connexin43 (Cx43). Adult rat hearts perfused on a Langendorff apparatus were subjected to ischemia or ischemia/reperfusion. Changes in coupling were monitored by measuring whole-tissue resistance. Changes in the amount and distribution of phosphorylated and nonphosphorylated isoforms of Cx43 were measured by immunoblotting and confocal immunofluorescence microscopy using isoform-specific antibodies. In control hearts, virtually all Cx43 identified immunohistochemically at apparent intercellular junctions was phosphorylated. During ischemia, however, Cx43 underwent progressive dephosphorylation with a time course similar to that of electrical uncoupling. The total amount of Cx43 did not change, but progressive reduction in total Cx43 immunofluorescent signal and concomitant accumulation of nonphosphorylated Cx43 signal occurred at sites of intercellular junctions. Functional recovery during reperfusion was associated with increased levels of phosphorylated Cx43. These observations suggest that uncoupling induced by ischemia is associated with dephosphorylation of Cx43, accumulation of nonphosphorylated Cx43 within gap junctions, and translocation of Cx43 from gap junctions into intracellular pools. (Circ Res. 2000;87:656-662.)

Key Words: connexin43 • gap junctions • ischemia • uncoupling • phosphorylation • arrhythmias

Alternations in electrical coupling of ventricular myocytes play an important role in arrhythmogenesis in acute and chronic ischemic heart disease.\(^1\)–\(^3\) Mechanisms mediating uncoupling during ischemia are related to multiple pathophysiological processes including progressive increases in intracellular Ca\(^{2+}\) and H\(^+\) concentrations and accumulation of amphipathic lipid metabolites.\(^4\)–\(^8\) Other mechanisms could also promote uncoupling, however, and contribute to the development of conduction abnormalities and arrhythmias. Insights into mechanisms responsible for uncoupling could suggest novel therapeutic strategies to limit lethal arrhythmias induced by ischemia.

Intercellular electrical coupling channels are composed of connexins, members of a family of proteins that form gap junctions.\(^9\)–\(^10\) Studies of ventricular conduction in connexin43 (Cx43)–deficient mice have revealed that Cx43 is the principal ventricular electrical coupling protein.\(^11\)–\(^12\) Like many of the connexins, Cx43 is a phosphoprotein.\(^13\)–\(^15\) Changes in connexin phosphorylation can affect channel properties and connexin turnover dynamics.\(^14\)–\(^21\) Because acute ischemia may activate or inhibit protein kinases and phosphatases,\(^22\) we performed the present study to test the hypothesis that electrical uncoupling induced by myocardial ischemia is mediated, at least in part, by alterations in phosphorylation of Cx43. We studied an isolated rat heart preparation in which the time course of uncoupling during ischemia was similar to that reported in rabbit\(^23\) and porcine\(^24\) hearts. We observed that in response to ischemia, Cx43 underwent marked dephosphorylation with a time course similar to that of uncoupling. Uncoupling was associated with diminished Cx43 immunoreactive signal and concomitant accumulation of nonphosphorylated Cx43 at sites of intercellular connections. Recovery of contractile function after reperfusion was associated with increased levels of phosphorylated Cx43. These observations suggest that uncoupling induced by ischemia may be related to dephosphorylation of Cx43 within gap junctions and translocation of Cx43 from gap junctions to intracellular sites.
Materials and Methods

Animal Care
Animals were housed in barrier facilities under veterinary supervision. All protocols were approved by the Animal Studies Committee of Washington University School of Medicine.

Perfusion of Isolated Hearts
Hearts excised from adult male Sprague-Dawley rats were transferred to a Langendorff apparatus and perfused via an aortic cannula as previously described. After a 10-minute stabilization interval of normoxic perfusion, hearts were made ischemic by cessation of perfusion for 0 to 40 minutes. In some studies, hearts were subjected to global ischemia for 20 minutes and then reperfused with oxygenated buffer for 30 minutes.

Measurement of Whole-Tissue Resistance
The time course of electrical uncoupling induced by ischemia was characterized in 5 additional hearts by measuring changes in whole-tissue resistance, \( r_t \). This method has been validated in rabbit papillary muscle by cable analysis. Briefly, polytetrafluoroethylene (Teflon)–coated silver wire electrodes (0.045-inch coated diameter) were placed on the epicardial surface of the heart in an orientation roughly parallel to the long axis of the epicardial ventricular fibers. Electrical recordings were made at a point 50 μm below the epicardial surface where the Teflon insulation had been removed from each wire. The outer 2 electrodes, each separated from its adjacent inner electrode by a distance of 1.0 mm, were connected to a current source, and the inner 2 electrodes, separated from each other by a distance of 1.5 mm, were connected to a voltage amplifier. A subthreshold current of 20-ms duration was delivered across the outer 2 electrodes, and the voltage drop across the inner 2 electrodes was recorded. Tissue resistance, \( r_t \), is a measure of the resistance of the extracellular (\( r_e \)) and intracellular (\( r_i \)) spaces arranged in parallel (\( 1/r_t = 1/r_e + 1/r_i \)). Data were normalized to control measurements obtained during normoxic perfusion in each heart to permit comparisons between hearts. Once the electrodes had been placed, hearts were perfused with oxygenated buffer, and baseline \( r_t \) values were obtained during a preischemic perfusion period of 10 minutes. Hearts were then subjected to global ischemia, and \( r_t \) was measured every 2 minutes. During ischemia, \( r_t \) showed the characteristic time course defined by an immediate early rise (first phase, vascular collapse), a subsequent slow rise (second phase, rise in extracellular resistance), and a marked final rise (third phase, cell-to-cell uncoupling). The onset of uncoupling was determined in each experiment by the transition from the second to the third phase (see Figure 1, top).

Antibodies
A rabbit polyclonal antibody (Zymed) directed against epitopes in the C terminus of rat Cx43 was used in immunoblotting and immunofluorescence studies as described previously. We also used a mouse monoclonal antibody (Zymed) shown by Nagy et al to bind selectively to nonphosphorylated Cx43. As detailed below, we have confirmed this finding and used this antibody to characterize the amount and distribution of nonphosphorylated Cx43 in rat hearts subjected to ischemia.

Preparation and Quantification of Immunoblots
Hearts were removed from the perfusion apparatus, trimmed of atria and great vessels, and immediately freeze-clamped. Pulverized samples were prepared and analyzed by quantitative immunoblotting as described previously.

Immunofluorescence and Confocal Microscopy
Hearts were removed from the perfusion apparatus and fixed in 10% neutral buffered formalin in preparation for paraffin embedding and confocal immunohistochemical analysis as previously described. The amount of high-intensity Cx43 signal in discrete spots at intercellular junctions was measured as described previously and expressed as a proportion of total tissue area.

Results
Changes in \( r_t \) During Acute Global Ischemia
Figure 1 shows changes in \( r_t \) induced by ischemia. As shown previously with cable analysis in rabbit hearts, changes in \( r_t \) in no-flow ischemia occurred in characteristic phases. A rapid initial phase was associated with vascular collapse after induction of ischemia. Between 4 and 14 minutes, \( r_t \) increased slowly, presumably because of osmotic water shifts from the extracellular to the intracellular compartment as previously
The plateau in pattern was virtually identical to results of previous studies.22–24 The mean time of onset of uncoupling was 15.2 ± 3.3 minutes. The observed pattern was virtually identical to results of previous studies.3,23,24 The plateau in r, seen ≈22 minutes after the onset of uncoupling indicated that complete uncoupling had occurred.29,30

**Characterization of Cx43 Phosphorylation Isoforms by Immunoblot Analysis**

The polyclonal anti-Cx43 antibody detected major bands at 44 and 46 kDa and a faint band at 41 kDa in a blot prepared from control rat ventricular homogenate (Figure 2), consistent with previous reports that most of the Cx43 in the heart is phosphorylated.20,28,31 When the homogenate was preincubated with alkaline phosphatase, only a single intense band at 41 kDa was observed. These results indicate that the higher molecular weight bands comprise phosphorylated isoforms of Cx43 and the rabbit polyclonal antibody recognizes both phosphorylated and nonphosphorylated isoforms on polyacrylamide gels. The mouse monoclonal anti-Cx43 antibody barely recognized a faint band at 41 kDa (Figure 2); it did not react with the more prominent, higher molecular weight bands seen with the polyclonal antibody. When the homogenate was preincubated with alkaline phosphatase, this antibody recognized an intense band at 41 kDa. These results confirm that the mouse monoclonal antibody selectively binds nonphosphorylated Cx43.

**Changes in Cx43 Phosphorylation During Acute Ischemia and Ischemia/Reperfusion**

Isolated perfused rat hearts were subjected to global ischemia for 15, 30, or 40 minutes. The 15-minute time point corresponded to the average time of onset of cell-to-cell uncoupling, and the 30- and 40-minute points were times of more advanced and full uncoupling, respectively (see Figure 1). Figure 3A shows a representative immunoblot prepared with the polyclonal antibody. Acute ischemia was associated with marked loss of phosphorylated Cx43 (bands at 44 to 46 kDa) and a corresponding increase in nonphosphorylated Cx43 (41-kDa signal). Loss of phosphorylated Cx43 was apparent after 15 minutes of ischemia when uncoupling had just begun and became more marked after 30 or 40 minutes of ischemia. Densitometric analysis of immunoblots from 4 hearts at each time point (Figure 3B) revealed no change in the total amount of Cx43 signal (phosphorylated plus nonphosphorylated isoforms). Thus, a 40-minute interval of global ischemia is associated with progressive dephosphorylation of Cx43 but no net loss of total Cx43 protein content from ventricular myocardium.

To further characterize the temporal relationship between uncoupling and Cx43 dephosphorylation, we prepared immunoblots from 5 hearts that had undergone ischemia for 7 minutes. At this time, all hearts had ceased contractile activity but electrical uncoupling had not yet begun, as shown in Figure 1 and indicated by the mean time of onset of uncoupling (15.2 ± 3.3 minutes; n = 5). As shown in Figure 4A, there was little change in the relative proportions of phosphorylated and nonphosphorylated Cx43 in 3 representative hearts made globally ischemic for 7 minutes.

To determine whether reperfusion after ischemia led to reaccumulation of phosphorylated Cx43, we studied hearts subjected to 20 minutes of ischemia followed by reperfusion for 30 minutes. We selected 20 minutes of ischemia because by this time, electrical uncoupling and Cx43 dephosphorylation had begun (see Figures 1 and 3). Furthermore, some hearts resumed contractile activity during a subsequent 30-minute interval of reperfusion, whereas others failed to resume contractions and had presumably become irreversibly injured. This ischemia/reperfusion protocol was, therefore, well suited to investigating the relationship between functional recovery after ischemia and phosphorylation of Cx43. We monitored the return of contractile activity rather than a decrease in r, as an indicator of functional recovery because in preliminary studies we sometimes observed a rapid,
dramatic fall in $r_t$ toward the baseline value after reperfusion even when no contractile function had returned. This rapid decrease in $r_t$ is apparently related to disruption of myocyte membranes and/or to marked extracellular edema after reperfusion, each of which interferes with maintenance of separate extracellular and intracellular resistive components in the electrical bidomain.3

Figures 4B and 4C show immunoblots from hearts that had undergone 20 minutes of ischemia or 20 minutes of ischemia followed by 30 minutes of reperfusion (lanes 4 through 7) compared with a control heart (lane 1). The blot was probed with polyclonal anti-Cx43 antibody. C, The same blot shown in panel B after being stripped and reprobed with a mouse monoclonal anti-Cx43 antibody. In each blot, upper and lower arrows indicate the positions of the 46- and 41-kDa bands, respectively.

Changes in the Distribution of Cx43 Isoforms During Acute Ischemia

Figure 5 shows representative confocal images from a control heart and hearts subjected to global ischemia. Figure 6 shows quantitative digital image analysis of the proportion of tissue area occupied by discrete spots of intense immunofluorescent signal in 5 fields per heart from 4 hearts at each time point. In sections stained with the polyclonal antibody, intense immunofluorescent signal was seen in control hearts at discrete sites of intercellular apposition. In contrast, virtually no signal was seen in control myocardium stained with the monoclonal antibody. This observation confirms previous findings suggesting that most if not all of the Cx43 in gap junctions in normal myocardium is phosphorylated.20,28,31 With ischemia, progressive loss of immunoreactive signal at apparent gap junctions occurred in sections stained with the monoclonal antibody, a corresponding increase in signal strength, and a marked difference in the level of nonphosphorylated Cx43 in reperfused hearts that had recovered contractile function and those that were irreversibly injured. Taken together, these results suggest that significant dephosphorylation of Cx43 does not occur until the tissue uncouples, whereas reversal of uncoupling with reperfusion, reflected by a recovery of contractile function, is associated with reaccumulation of phosphorylated Cx43.

Figure 4. A, Immunoblot of Cx43 in hearts subjected to 7 minutes of ischemia (lanes 2 through 4) and a control heart (lane 1) probed with polyclonal anti-Cx43 antibody. B, Immunoblot of Cx43 in hearts subjected to 20 minutes of ischemia (lanes 2 and 3) or 20 minutes of ischemia and 30 minutes of reperfusion (lanes 4 through 7) compared with a control heart (lane 1). The blot was probed with polyclonal anti-Cx43 antibody. C, The same blot shown in panel B after being stripped and reprobed with a mouse monoclonal anti-Cx43 antibody. In each blot, upper and lower arrows indicate the positions of the 46- and 41-kDa bands, respectively.
in sections stained with the monoclonal antibody. Because the relative titers and binding affinities of the 2 anti-Cx43 antibodies used in these studies are not known, it is not possible to directly compare the relative amounts of phosphorylated and nonphosphorylated Cx43 in gap junctions at any point during ischemia. Nevertheless, the results in Figures 5 and 6 clearly indicate that during uncoupling, nonphosphorylated Cx43 accumulated in sites of intercellular apposition (presumed gap junctions) while at the same time the amount of total Cx43 (phosphorylated and nonphosphorylated) in gap junctions was progressively reduced. The concentration of intracellular Cx43 was sufficiently low that it was not detected by the image-processing method used to analyze signal in intercellular junctions. However, because the total tissue content of Cx43 did not change during ischemia (Figure 3B), the immunofluorescence data indicate that Cx43 translocates from gap junctions to intracellular site(s) during uncoupling.

Qualitative immunofluorescence analysis was performed on representative hearts subjected to 20 minutes of ischemia followed by 30 minutes of reperfusion. As shown in Figure 7, the amount of total Cx43 signal was dramatically reduced and the amount of nonphosphorylated Cx43 signal was greatly increased in hearts that failed to recover contractile function during reperfusion (Figure 3B), the immunofluorescence data indicate that Cx43 translocates from gap junctions to intracellular site(s) during uncoupling.

Discussion

The results of this study show that during the process of electrical uncoupling induced by ischemia, the principal cardiac gap-junction channel protein, Cx43, undergoes marked dephosphorylation. In previous studies, perfusion of isolated rat hearts with oxygenated buffer for 4 hours caused no change in Cx43 phosphorylation.20 Thus, dephosphorylation of Cx43 in the present studies was not related to the isolated perfused heart preparation. Although measurement of r, provides only a qualitative index of cell-to-cell coupling, the different phases of uncoupling during ischemia can be clearly discerned. With this approach, uncoupling and Cx43 dephosphorylation were first detected at about the same time after the onset of ischemia and progressed with a similar time course. Reperfusion after an interval of ischemia sufficient to cause both uncoupling and Cx43 dephosphorylation led to at least partial restoration of the control level of phosphorylated Cx43 in hearts that recovered contractile activity, whereas further dephosphorylation of Cx43 occurred in hearts that failed to recover. Immunohistochemical analysis using isoform-selective antibodies revealed that under physiological conditions, nearly all Cx43 at sites of intercellular apposition was phosphorylated. With uncoupling, however, there was progressive loss of Cx43 signal at sites of intracellular apposition concomitant with a marked increase in nonphosphorylated Cx43. In light of immunoblot data showing that the total amount of Cx43 remained constant throughout the ischemic interval, these observations suggest that uncoupling resulted in progressive dephosphorylation of Cx43, accumulation of the nonphosphorylated isoform within gap junctions, and translocation of Cx43 from gap junctions into an intracellular pool.

Although abundant evidence indicates that phosphorylated and nonphosphorylated isoforms of Cx43 migrate on SDS-polyacrylamide gels at 44 to 46 and 41 kDa, respectively, specific phospho–amino acid residues and the diversity of phosphorylated isoforms of Cx43 in the heart have not been defined precisely. Shifts in the migration of Cx43 bands and changes in the intensity of immunoreactive signal detected by antibodies used in the present study indicated that marked alterations in the amount and distribution of phosphorylated Cx43 occurred during ischemia, but no conclusions can be
reached about specific biochemical changes in Cx43. It is possible that the monoclonal antibody could have detected some phosphorylated isoforms of Cx43 that migrated with apparent molecular weights at or near 41 kDa.32

Multiple mechanisms likely contribute to uncoupling during ischemia, including increased levels of intracellular Ca2+, progressive intracellular acidosis,33,34 accumulation of lipid metabolites that might act as uncoupling agents,35,36 and other potential mechanisms. Our results provide new evidence that rapid, reversible Cx43 dephosphorylation could also contribute to myocardial uncoupling and, thereby, play a role in arrhythmogenesis during acute ischemia. One potential mechanism of ischemia-induced accumulation of dephosphorylated Cx43 is decreased intracellular ATP concentration and decreased thermodynamic force for phosphorylation (the free-energy change of ATP hydrolysis, ΔGATP). Interestingly, the decrease in ΔATP during ischemia is biphasic with a moderate immediate decrease and a marked secondary decrease that coincides with cell-to-cell uncoupling.33 Although it remains unknown whether uncoupling is preceded and caused by Cx43 dephosphorylation, whether dephosphorylation occurs in channels that have already uncoupled, or whether Cx43 dephosphorylation is the result of increased phosphatase and/or decreased kinase activity, it is likely that a reduction in ΔATP shifts the thermodynamic equilibrium toward dephosphorylation. In any case, dephosphorylation could serve as an initial step in translocation of Cx43 from gap junctions into the cytoplasm during ischemia. The biological consequences of specific changes in Cx43 phosphorylation are not understood in detail. Dramatic decreases in gap junctional communication, and concomitant loss of phosphorylated Cx43 and accumulation of nonphosphorylated Cx43 have been reported in epithelial cells treated with 18β-glycyrrhetinic acid34 and glial cells exposed to oleamide29 but, as in the present study, the relationship between changes in phosphorylation at specific residues and uncoupling was not defined. Although phosphorylation of serine residues in the carboxyl-terminal intracellular domain of Cx43 appears to be the major post-translation modification in Cx43 migrating on gels at 44 to 46 kDa,14,33 and generalized loss of serine phosphorylation reflected by a shift to 41 kDa is associated with uncoupling,34,35 phosphorylation of tyrosine residues can occur in Cx43 and is also associated with uncoupling.15,17,36 It is not known whether tyrosine phosphorylation of Cx43 occurred during uncoupling induced by ischemia in the present studies or whether the monoclonal antibody detected Cx43 isoforms containing phosphoryrosine. Future studies will be required to define the precise pathophysiological relationship between changes in phosphorylation at specific amino acid residues of Cx43 and uncoupling and the potential effects of modulating Cx43 phosphorylation on the development of arrhythmias during ischemia.

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