Mechanisms of Delayed Electrical Uncoupling Induced by Ischemic Preconditioning

Sandeep K. Jain, Richard B. Schuessler, Jeffrey E. Saffitz

Abstract—Electrical uncoupling of cardiac myocytes during ischemia is delayed by ischemic preconditioning. This presumably adaptive response may limit development of arrhythmia substrates. To elucidate responsible mechanisms, we studied isolated, perfused rat hearts subjected to a standard preconditioning protocol of 3 cycles of 3 minutes of global no-flow ischemia each followed by 5 minutes of reperfusion before a 30-minute interval of ischemia. Changes in coupling were monitored by measuring whole-tissue resistance. Changes in phosphorylation and subcellular distribution of connexin43 (Cx43) were defined by quantitative immunoblotting and confocal microscopy. Preconditioning caused a 34% decrease in the maximal rate of uncoupling and delayed the time to plateau in uncoupling. Dephosphorylation of Cx43, known to occur during uncoupling induced by ischemia, was dramatically decreased in preconditioned hearts. Translocation of Cx43 from gap junctions to the cytosol, also known to occur during ischemia, was reduced by >5-fold in preconditioned hearts. The K<sub>ATP</sub> channel blockers glybenclamide and 5-hydroxydecanoate prevented these effects in preconditioned hearts, whereas the K<sub>ATP</sub> channel agonist diazoxide mimicked these effects in nonpreconditioned hearts. Intracellular translocation of Cx43 was blocked, but Cx43 dephosphorylation was not blocked during ischemia in preconditioned hearts treated with the PKC inhibitors chelerythrine and calphostin C. Uncoupling during ischemia was accelerated by PKC and K<sub>ATP</sub> channel inhibition. Thus, delayed uncoupling in preconditioned hearts is likely related to diminished dephosphorylation and intracellular redistribution of Cx43 during prolonged ischemia. Both of these effects are regulated by activation of K<sub>ATP</sub> channels, whereas PKC plays a role in internalization of Cx43. (Circ Res. 2003;92:1138-1144.)

Key Words: preconditioning ▪ gap junctions ▪ connexin43 ▪ coupling

Ischemic preconditioning produced by brief, repetitive episodes of ischemia protects the heart from injury during a subsequent bout of sustained ischemia. The best characterized salutary effect of ischemic preconditioning is reduced infarct size, which has been well documented in many species. Preconditioning also protects against development of arrhythmias, but this effect has not been as thoroughly investigated as the effect on infarct size. Reperfusion arrhythmias have been shown to be decreased in an in vivo rat model of ischemic preconditioning, and recently, diminished postoperative arrhythmias have been observed in patients who were treated with a preconditioning protocol before undergoing coronary artery bypass surgery. The mechanism by which arrhythmia protection occurs has not been studied in detail.

Several lines of evidence suggest that electrical uncoupling during acute ischemia plays a key role in arrhythmogenesis, and that gap junction channels may be an important target in ischemic preconditioning. For example, mice with genetic deficiency in connexin43 (Cx43), the major ventricular gap junction protein, exhibit a significantly greater incidence, frequency, and duration of spontaneous and inducible arrhythmias than wild-type littermates after coronary occlusion. Thus, it appears likely that during electrical uncoupling in the setting of acute ischemia, there may be a particularly vulnerable interval in which conduction is still maintained but is sufficiently slow and/or heterogeneously deranged such that the risk of reentry becomes high. Moreover, Tan et al and Cinca et al have shown that ischemic preconditioning delays electrical uncoupling during ischemia. In the complex dynamics of ischemic injury, even a modest delay in uncoupling could have salutary effects on the development of acute arrhythmia substrates. Little is known, however, about potential mechanisms responsible for delayed uncoupling after preconditioning.

We have previously demonstrated that electrical uncoupling induced by ischemia is associated with marked dephosphorylation of Cx43 and translocation of Cx43 from gap junctions to intracellular stores. To elucidate potential mechanisms by which ischemic preconditioning delays uncoupling, we characterized changes in Cx43 phosphorylation and subcellular distribution in an isolated rat heart prepara-
tion subjected to a classical preconditioning protocol followed by an interval of global ischemia sufficient to produce uncoupling. We confirmed previous studies showing that preconditioning delays uncoupling.9,10 We also observed that preconditioning markedly retards the intracellular translocation of Cx43 and greatly diminishes Cx43 dephosphorylation during ischemia. Finally, we found that changes in the kinetics of uncoupling and the subcellular distribution and phosphorylation state of Cx43 are regulated by activation of ATP-sensitive potassium (K\textsubscript{ATP}) channels and protein kinase C (PKC) during preconditioning.

Materials and Methods

Animal Studies

All experiments involving animals were approved by the Animal Studies Committee at Washington University School of Medicine.

Isolated Heart Perfusion

Hearts of anesthetized adult male Sprague-Dawley rats (300 to 325 g) were excised, transferred to a Langendorff apparatus, and perfused via aortic cannula with Krebs-Henseleit buffer at 37°C in the nonrecirculating mode as previously described.11 Initial flow was adjusted to achieve a retrograde perfusion pressure of 50 to 80 mm Hg. Flow rate was then kept constant for the remainder of the experiment. In some experiments, a water-filled latex balloon connected to a pressure transducer was placed in the left ventricle for continuous monitoring of left ventricular pressure. All hearts were initially perfused with oxygenated buffer during a 15-minute stabilization period. Hearts were then subjected to a preconditioning protocol consisting of 3 cycles of 3 minutes of global no-flow ischemia followed by 5 minutes of normal perfusion before undergoing a 30-minute interval of global ischemia. Control (nonpreconditioned) hearts were subjected to 30 minutes of no-flow ischemia without undergoing prior preconditioning.

Pharmacological Agents

Chelerythrine chloride (Sigma, 5 \mu\text{mol/L}), calphostin C (Calbiochem, 200 \mu\text{mol/L}), glibenclamide (Sigma, 10 \mu\text{mol/L}), and 5-hydroxydecanoate (Sigma, 200 \mu\text{mol/L}) were administered 3 minutes before and continued throughout the preconditioning protocol. Diazoxxe (Sigma, 100 \mu\text{mol/L}) was administered for 10 minutes before the onset of prolonged ischemia.

Measurement of Whole-Tissue Resistance and Parameters of Electrical Uncoupling

Electrical uncoupling during ischemia was monitored by measuring changes in whole-tissue resistance using the 4-electrode method.12-14 Once perfusion with normoxic buffer had been initiated in excised hearts, 4 Teflon-coated silver wire electrodes (0.045-in coated diameter) were passed through the anterior surface of the left ventricle in a linear arrangement oriented parallel to the long axis of epicardial fibers. The tip of each wire contained a bead of epoxy to prevent the wire from pulling through the epicardial surface. The Teflon insulation had been removed at a point \approx 50 \mu\text{m} below the bead to permit measurements at a consistent intramyocardial position below the epicardial surface. The outer two 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 alternating current (1000Hz; peak to peak amplitude 19 \mu\text{A}) was delivered across the outer two electrodes while the voltage drop across the inner two electrodes was recorded. Tissue resistance ($r_t$), a measure of extracellular ($r_e$) and intracellular ($r_i$) resistances arranged in parallel ($1/r_t = 1/r_e + 1/r_i$), was measured at 1-min intervals throughout the experiment.

Tissue resistance data were normalized to control values obtained during normoxic perfusion to permit comparisons between hearts (initial $r_t$ values were set to 1.0 in each experiment). The onset of uncoupling was defined as the time of an initial 3% increase in $r_t$ from the prior measurement, which then continued to increase. Plateau time was defined as the time at which the first of 3 consecutive identical $r_t$ measurements was observed. The maximum slope was calculated by linear regression during the steep rise in the uncoupling curve using a minimum of 5 points. Fold increase in $r_t$ was the absolute resistance increase from baseline to plateau.

Quantitative Confocal Immunofluorescence Microscopy and Immunoblotting

A rabbit polyclonal antibody (Zymed) directed against unique epitopes in the C-terminus of rat Cx43 was used in immunoblotting and immunofluorescence studies as described previously.15,16 In preparation for confocal microscopy, hearts were removed from the perfusion apparatus, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 5 \mu\text{m}. Slide-mounted sections were incubated overnight with the anti-Cx43 antibody (diluted 1:100) and then with Cy3-conjugated goat anti-rabbit IgG (diluted 1:400) before being examined by laser scanning confocal microscopy. The amount of Cx43 signal at intercellular junctions was quantified as described previously and expressed as a proportion of total tissue area. In preparation for immunoblot analysis, hearts were removed from the perfusion apparatus, the atria and great vessels were discarded, and tissue was frozen for subsequent analysis as previously described.15,16

Statistical Analysis

All data are expressed as mean ± SD except where otherwise stated. Differences between groups were analyzed with ANOVA and Fisher’s protected least significant difference test. A value of $P<0.05$ was considered statistically significant.

Results

Validation of the Preconditioning Protocol

To confirm that the preconditioning protocol was effective, we compared recovery of LV developed pressures in preconditioned and nonpreconditioned hearts exposed to 30 minutes of global ischemia followed by 30 minutes of reperfusion. At the end of reperfusion, there was a significant improvement in recovery of contractile function in preconditioned hearts (95.7 ± 2.7% versus 45.1 ± 29.9% of baseline LV developed pressure, n=5 in each group; $P<0.02$).

![Figure 1](https://example.com/image1.png)

**Figure 1.** Whole-tissue resistance curves measured in preconditioned (PC, n=20) and nonpreconditioned (Non-PC, n=15) hearts subjected to global ischemia to induce uncoupling. Resistance measurements have been normalized to allow comparisons between experiments. Error bars represent standard error of the mean.
Ischemic Preconditioning Delays Uncoupling During Prolonged Ischemia

To evaluate the effects of ischemic preconditioning on uncoupling during a subsequent interval of ischemia, we measured changes in whole-tissue resistance ($r_t$) using the 4-electrode method in preconditioned and nonpreconditioned hearts. Previous studies have established that during ischemia, $r_t$ increases in characteristic phases and that the sustained rise in $r_t$ seen after a modest, transient rise in $r_t$ is a direct result of electrical uncoupling at gap junctions. As shown in Figure 1 and the Table, preconditioning decreased the maximal rate of uncoupling during ischemia by 34% (determined by measuring the slopes of the $r_t$ curves during the rapid uncoupling phase). Preconditioning also significantly increased the time required to reach a plateau in $r_t$. The onset of uncoupling occurred later in preconditioned hearts, but relatively large standard deviations prevented this difference from achieving significance. Although preconditioned hearts eventually reached a maximum level of uncoupling equal to that of nonpreconditioned hearts, preconditioning significantly slowed the rate of uncoupling and lengthened the time required for complete uncoupling.

Preconditioning Prevents Dephosphorylation and Intracellular Redistribution of Cx43

To determine whether delayed uncoupling during ischemia induced by preconditioning is associated with changes in Cx43 phosphorylation and subcellular distribution, preconditioned and nonpreconditioned hearts were subjected to 30 minutes of global ischemia and then analyzed by confocal immunofluorescence microscopy and immunoblotting using an anti-Cx43 antibody that binds to both phosphorylated and nonphosphorylated isoforms of Cx43. As previously observed, the amount of Cx43 immunoreactive signal in gap

![Figure 2. Top, Representative confocal microscopy images showing the amount of Cx43 immunoreactive signal at cell-cell junctions in a control heart and in hearts exposed to 30 minutes of ischemia without or with a preconditioning period. Bottom, Quantitative confocal microscopy measurements showing the amount of Cx43 signal expressed as a percent of total tissue area in control hearts analyzed immediately after euthanasia (CTRL), control hearts subjected to 70 minutes of normal perfusion (CTRL-Perfused), and hearts subjected to 30 minutes of global ischemia without (Non-PC) or with (PC) a preconditioning period. Hatched bars show the amount of Cx43 signal in nonpreconditioned or preconditioned hearts after 30 minutes ischemia followed by 30 minutes of reperfusion. n=6 for each group. *P<0.003 vs PC; †P<0.011 vs PC.]}
junctons was greatly reduced after 30 minutes of ischemia in hearts that did not undergo preconditioning (Figure 2). In contrast, preconditioning resulted in a marked preservation of Cx43 signal in gap junctions. The amount of signal at intercellular junctions was more than 5-fold greater in preconditioned hearts compared with nonpreconditioned hearts that underwent 30 minutes of ischemia (Figure 2). The amount of Cx43 signal increased during reperfusion in both preconditioned and nonpretreconditioned hearts, but preconditioned hearts still had a significantly greater amount of Cx43 signal in gap junctions after 30 minutes of reperfusion (Figure 2).

To measure the total amount of Cx43 in ventricles of preconditioned and nonpreconditioned hearts subjected to ischemia and to assess the effects of preconditioning on Cx43 phosphorylation, ventricular tissues were analyzed by immunoblotting. The polyclonal anti-Cx43 antibody used in these studies is known to bind to phosphorylated and nonphosphorylated isoforms of Cx43. As reported previously, there was a clear shift from phosphorylated to nonphosphorylated forms of Cx43 in hearts that experienced 30 minutes of ischemia without prior preconditioning (Figure 3). However, in hearts that were preconditioned, minimal dephosphorylation could be detected (Figure 3) even though these hearts also experienced 30 minutes of ischemia. Densitometric analysis of immunoblots showed equal amounts of Cx43 in ventricular lysates from perfused hearts that had never been ischemic and in preconditioned and nonpreconditioned hearts that underwent 30 minutes of ischemia (Figure 3). The preconditioning protocol itself did not change Cx43 distribution or phosphorylation state (data not shown). Taken together, the data in Figures 2 and 3 show that preconditioning dramatically reduces dephosphorylation of Cx43 and retards the translocation of Cx43 from gap junctions to intracellular loci that normally occurs during an interval of ischemia sufficient to lead to electrical uncoupling.

Figure 3. Top, Representative immunoblot of Cx43 in whole ventricular lysates prepared from a perfused control heart (CTRL-P) and from hearts subjected to 30 minutes of ischemia without (Non-PC) or with (PC) a preconditioning period. The nonpreconditioned heart shows marked dephosphorylation of Cx43 indicated by a shift toward lower molecular weight forms and accumulation of signal at 41 kDa, the position of nonphosphorylated Cx43. No dephosphorylation is detectible in the preconditioned heart. Bottom. Densitometric measurements of total Cx43 signal (phosphorylated + nonphosphorylated) in control hearts processed immediately after euthanasia (CTRL, n=3), control hearts subjected to normal perfusion for 70 minutes (CTRL-P, n=3), and hearts subjected to 30 minutes of ischemia without (Non-PC, n=6) or with (PC, n=6) a previous preconditioning period. No significant change in total Cx43 content is apparent in any group.

Preconditioning-Induced Changes in Cx43 and Uncoupling Are Mediated by Activation of K<sub>ATP</sub> Channels and PKC

Salutary effects of ischemic preconditioning on myocardial infarct size are mediated, at least in part, by activation of protein kinase C (PKC) and ATP-sensitive potassium (K<sub>ATP</sub>) channels. To determine whether these factors play a role in the effects of preconditioning on Cx43, we characterized the effects of inhibitors of K<sub>ATP</sub> channel activation and PKC on Cx43 phosphorylation and distribution. Addition of the K<sub>ATP</sub> channel blockers glybenclamide (10 μmol/L) or 5-hydroxydecanoate (200 μmol/L) 3 minutes before and during the preconditioning protocol strongly inhibited the effects of preconditioning on Cx43 signal in gap junctions, whereas the K<sub>ATP</sub> channel agonist diazoxide (100 μmol/L) mimicked these effects in nonpreconditioned hearts. Thus, despite preconditioning, hearts treated with K<sub>ATP</sub> channel blockers showed marked loss of Cx43 signal in gap junctions, but Cx43 signal was strongly preserved in nonpreconditioned hearts treated with a K<sub>ATP</sub> channel agonist (Figure 4). Similar results were seen at a lower dose of 5-hydroxydecanoate (150 μmol/L) (data not shown). Inhibition of K<sub>ATP</sub> channel activation also blocked the effects of preconditioning on Cx43 phosphorylation (Figure 4). Thus, despite preconditioning, there was marked dephosphorylation of Cx43, whereas only limited Cx43 dephosphorylation was seen in nonpreconditioned hearts treated with diazoxide (Figure 4). These results indicate that activation of K<sub>ATP</sub> channels during preconditioning limits both dephosphorylation and intracellular redistribution of Cx43 during 30 minutes of no-flow ischemia.

Similar experiments were performed using the PKC inhibitors chelerythrine (5 μmol/L) or calphostin C (200 nmol/L). The use of these inhibitors in preconditioned hearts had no apparent effect on Cx43 signal in junctions. Equivalent amounts of Cx43 were seen in preconditioned hearts that were or were not treated with PKC inhibitors before being subjected to 30 minutes of ischemia (Figure 5). Furthermore, inhibition of PKC with chelerythrine completely blocked internalization of Cx43 in nonpreconditioned hearts subjected to ischemia (Figure 5). However, in both preconditioned and nonpreconditioned hearts treated with PKC inhibitors, Cx43 became dephosphorylated during ischemia (Figure 5). These results indicate that dephosphorylation of Cx43 can precede
and occur independently of intracellular redistribution of Cx43, and that activation of PKC is required for internalization of Cx43 during ischemia.

To determine whether inhibition of K<sub>ATP</sub> channels and/or PKC altered the effects of preconditioning on the time course of uncoupling, we measured changes in r<sub>i</sub> in hearts treated with inhibitors during preconditioning and then subjected to global ischemia. Inhibition of K<sub>ATP</sub> channels or PKC greatly accelerated uncoupling. Preconditioned hearts treated with either glybenclamide, 5-hydroxydecanoate, or chelerythrine began to uncouple significantly earlier and reached a plateau in uncoupling significantly sooner than preconditioned hearts not treated with inhibitors (Table). Furthermore, the onset of uncoupling occurred later in non-preconditioned hearts treated with diazoxide (Table). Taken together, these results indicate that activation of PKC and K<sub>ATP</sub> channels in preconditioned hearts delays uncoupling during ischemia.

**Discussion**

The results of this study indicate that ischemic preconditioning markedly retards intracellular translocation of Cx43 in gap junctions and significantly diminishes dephosphorylation of Cx43 during a subsequent bout of prolonged ischemia. We also confirmed previous studies<sup>8,9</sup> showing that preconditioning delays electrical uncoupling induced by ischemia. Taken together, these results suggest that preservation of phosphorylated Cx43 in gap junctions is responsible for delayed uncoupling induced by preconditioning.

Multiple signaling pathways have been implicated in the limitation of infarct size in ischemic preconditioning includ-
ing those activated or mediated by adenosine, bradykinin, protein kinase C, ATP-sensitive potassium channels, and tyrosine kinase. In the present study, we found that changes induced by preconditioning in the onset of uncoupling and in the subcellular distribution and phosphorylation state of Cx43 are also regulated by activation of PKC and K\textsubscript{ATP} channels, but the effects of these pathways on Cx43 distribution and phosphorylation differed. Inhibition of K\textsubscript{ATP} channels abrogated the effects of preconditioning on both Cx43 distribution and phosphorylation. In contrast, although Cx43 apparently remained within cell-cell junctions in preconditioned hearts treated with PKC inhibition, it still underwent marked dephosphorylation, and under these conditions, uncoupling occurred significantly earlier in response to ischemia. PKC activation is, therefore, required for internalization of dephosphorylated Cx43 during ischemia but does not play a direct role in Cx43 dephosphorylation itself, which presumably involves activation of phosphatases that remove phosphates from multiple serine residues in the C-terminus of Cx43. Although our data clearly show that Cx43 undergoes marked dephosphorylation during ischemia, it is entirely possible that C-terminal serine residues in Cx43 undergo concomitant phosphorylation by PKC, which could act as a signal for internalization. This hypothesis and our observations concerning PKC are consistent with previous studies by Lampe et al showing that phosphorylation of Cx43 by PKC leads to closure of channels and subsequent internalization of Cx43 in gap junctions. It is unlikely, however, that phosphorylation of Cx43 by PKC would be detected as a clear shift in gel mobility.

Previous reports indicate that activation of K\textsubscript{ATP} channels occurs downstream of PKC in preconditioning. Mitochondrial K\textsubscript{ATP} channel activation has also been shown to alter cardiac mitochondrial function, including attenuation of mitochondrial Ca\textsuperscript{2+} overload as well as release of membrane proteins such as cytochrome c and adenylate kinase. Our data suggest that K\textsubscript{ATP} channel activation either directly or indirectly inhibits activation of phosphatases that dephosphorylate Cx43 during ischemia. This results in less dephosphorylated Cx43 in gap junctions that can be acted on by PKC and internalized, thereby preserving functional Cx43 in preconditioned hearts. This model accounts for the observations that K\textsubscript{ATP} channel inhibition during preconditioning caused marked dephosphorylation and internalization of Cx43, whereas PKC inhibition allowed Cx43 to become dephosphorylated but prevented internalization of Cx43.

The pathophysiological effects of changes in Cx43 induced by ischemic preconditioning are not clearly defined. A recent study has shown that preconditioning limits infarct size in wild-type mice but not in Cx43-deficient mice, suggesting that maintenance of a certain level of coupling is important in the preconditioning effect. We would further suggest that the preconditioning-induced delay in uncoupling could significantly reduce development of arrhythmias during ischemia and reperfusion. Although the extent of the delay in uncoupling was relatively modest, it occurred during a critical time in the development of acute arrhythmia substrates. Preservation of conduction in acutely injured tissue could diminish the likelihood of reentry and, thereby, reduce the incidence of ventricular tachycardias dependent on this mechanism. Unfortunately, the experimental protocols used in the present study are not well suited to a critical analysis of this question. Cessation of perfusion to produce global (no-flow) ischemia in isolated hearts leads rather quickly to asystole and does not, therefore, provide an appropriate setting in which to study arrhythmias arising in response to acute regional ischemia. Further studies will be necessary to define the exact role of preconditioning-induced changes in gap junctional coupling in arrhythmia protection.

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