Protein kinase Cε mediates salutary effects on electrical coupling induced by ischemic preconditioning

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BACKGROUND Ischemic preconditioning delays the onset of electrical uncoupling and prevents loss of the primary ventricular gap junction protein connexin 43 (Cx43) from gap junctions during subsequent ischemia.

OBJECTIVE To test the hypothesis that these effects are mediated by protein kinase C epsilon (PKCε), we studied isolated Langendorff-perfused hearts from mice with homozygous germline deletion of PKCε (PKCε-KO).

METHODS Cx43 phosphorylation and distribution were measured by quantitative immunoblotting and confocal microscopy. Changes in electrical coupling were monitored using the 4-electrode technique to measure whole-tissue resistivity.

RESULTS The amount of Cx43 located in gap junctions, measured by confocal microscopy under basal conditions, was significantly greater in PKCε-KO hearts compared with wild-type, but total Cx43 content measured by immunoblotting was not different. These unanticipated results indicate that PKCε regulates subcellular distribution of Cx43 under normal conditions. Preconditioning prevented loss of Cx43 from gap junctions during ischemia in wild-type but not PKCε-KO hearts. Specific activation of PKCε, but not PKCδ, also prevented ischemia-induced loss of Cx43 from gap junctions. Preconditioning delayed the onset of uncoupling in wild-type but hastened uncoupling in PKCε-KO hearts. Cx43 phosphorylation at the PKC site Ser368 increased 5-fold after ischemia in wild-type hearts, and surprisingly, by nearly 10-fold in PKCε-KO hearts. Preconditioning prevented phosphorylation of Cx43 in gap junction plaques at Ser368 in wild-type but not PKCε-KO hearts.

CONCLUSION Taken together, these results indicate that PKCε plays a critical role in preconditioning to preserve Cx43 signal in gap junctions and delay electrical uncoupling during ischemia.

KEYWORDS Connexin 43; Coupling; Gap junctions; Preconditioning; Protein kinase C

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Ischemic preconditioning, defined as brief intervals of ischemia/reperfusion preceding sustained ischemia, reduces infarct size[1–3] and prevents reperfusion arrhythmias.[4,5] Although the antiarrhythmic mechanism of preconditioning remains unknown, previous studies from our laboratory and others have shown that preconditioning delays electrical uncoupling of the myocardium during ischemia.[6–8] Preconditioning also prevents ischemia-induced changes in the distribution[7] and phosphorylation[7,9,10] of connexin 43 (Cx43), the primary ventricular gap junction protein. Cx43 is a critical target in ischemia and preconditioning, as evidenced by the fact that mice deficient in Cx43 show greater incidence of arrhythmias after coronary occlusion[11] and loss of preconditioning-induced cardioprotection,[12] a finding also observed in myocytes isolated from Cx43-deficient hearts subjected to simulated ischemia/reperfusion.[13]

Protein kinase C (PKC) is a superfamily of serine/threonine protein kinases implicated in preconditioning.[14–16] Specifically, the Ca^{2+}-independent PKC epsilon isoform (PKCε) is thought to play a critical role in preconditioning, as salutary effects of preconditioning on infarct size are absent in mice lacking PKCε.[17,18] Conversely, specific activation of PKCε before sustained ischemia mimics preconditioning[19,20] and prevents reperfusion arrhythmias.[20,21] We have previously shown that nonselective inhibition of PKC by chelerythrine or calphostin C blocks the ability of preconditioning to delay uncoupling and prevent Cx43 redistribution,[7] suggesting an important role for PKC in mediating the effects of preconditioning on gap junctions. Furthermore, PKC phosphorylates the intracellular C-terminal domain of Cx43 at Ser368[22] and Ser262,[23] and these reactions are probably catalyzed at least partially by PKCε, which co-immunoprecipitates with Cx43.[24,25] Phosphorylation at Ser368 has been shown to alter channel selectivity[26] and gap junction assembly,[27] although the effects on cell communication in the intact heart are unclear.

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Accordingly, the goal of the present study was to determine whether PKCε is responsible for mediating changes in Cx43 distribution and phosphorylation and electrical coupling at gap junctions during ischemic preconditioning. We found that in genetically engineered mice lacking PKCε, preconditioning fails to preserve Cx43 in gap junctions and cellular uncoupling is actually accelerated during sustained ischemia. These results identify PKCε as a potential target for regulating Cx43 distribution and electrical coupling during sustained ischemia.

Methods

Animals

Mice homozygous for a germline deletion of the gene encoding PKCε (PKCε-KO) were originally produced by Khasar et al.[28] F1-generation C57BL/6J and 129SvJae heterozygous progeny (generous gift from Dr. Robert O. Messing) were intercrossed to generate F2-generation hybrid C57Bl/6Jx129SvJae wild-type and PKCε-KO littermates. Previous studies have shown that these mice lack responsiveness to preconditioning in the absence of any gross or functional abnormality at baseline compared to wild-type littermates.[18] All studies were performed in adult animals 12 to 20 weeks of age. Experimental protocols were approved by the Animal Studies Committee at Washington University School of Medicine.

Isolated heart perfusion

Hearts of anesthetized adult mice were excised rapidly, transferred to a Langendorff apparatus, and perfused via aortic cannula with oxygenated Krebs-Henseleit buffer containing (in mmol/l): NaCl 118.3, KCl 2.7, $MgSO_4$ 1.0, KH_2PO_4 1.4, $NaHCO_3$ 29.0, $CaCl_2$ 3.4, and glucose 10, with insulin 70 mU/l and BSA 0.4% at 37°C. Flow was adjusted to achieve a retrograde perfusion pressure of 40 to 50 mm Hg. All hearts were initially perfused with oxygenated buffer during a 10-min stabilization period. A subset of hearts was then subjected to a preconditioning protocol consisting of 3 cycles of 3 min of global no-flow ischemia followed by 5 min of normal perfusion before undergoing 30 min of global ischemia, as described previously.[7] Other hearts were not subjected to preconditioning but were instead perfused with normoxic buffer for 24 min before undergoing 30 min of global ischemia.

In separate studies, hearts were perfused for 10 min with normoxic buffer containing peptide activators (1 μM) of PKCε[19] (KAE1-1, KAI Pharmaceuticals, San Francisco, CA) or PKCδ[29] (KAD1-1, KAI Pharmaceuticals) or a nonspecific control peptide (C-1, KAI Pharmaceuticals) before undergoing 30 min of global ischemia.

Antibodies

Antibodies used in this study included a rabbit polyclonal antibody (Zymed, Carlsbad, CA) directed against epitopes in the C-terminus of rat Cx43 (immunoblotting, 1:5000 dilution); a mouse monoclonal anti-Cx43 antibody (Chemicon, (Temecula, CA), MAB3068) (immunohistochemistry and confocal microscopy, 1:400 dilution); a rabbit polyclonal anti-phospho-Cx43 antibody directed against Ser368 (Cell Signaling, Danvers, MA) (immunoblotting, 1:500 dilution; immunostaining, 1:100 dilution); rabbit polyclonal anti-phospho-Cx43 antibodies directed against Ser262, Ser279/282, Ser255, or Tyr265 (Santa Cruz, Santa Cruz, CA) (immunoblotting); a monoclonal anti-PKCε antibody (BD Biosciences, San Jose, CA) (immunoblotting, 1:250 dilution); a polyclonal anti-PKCδ antibody (Santa Cruz) (immunoblotting, 1:750 dilution); a monoclonal anti-GAPDH antibody (RDI) (immunoblotting, 1:5000 dilution), and a polyclonal anti-actin antibody (Santa Cruz) (immunoblotting, 1:1000 dilution).

Preparation and quantification of immunoblots

Hearts were removed from the perfusion apparatus and trimmed of atria and great vessels. Apical and basal portions of each ventricle were frozen separately for subsequent analysis. Apical samples were prepared for Cx43 immunoblotting and analyzed as described previously.[30] Cx43 band densities were divided by their respective glyceraldehyde 3-phosphate dehydrogenase (GAPDH) density values and then normalized to the same control sample on each gel. A subset of immunoblots was probed for actin to verify that 30 min of global ischemia does not alter GAPDH levels (data not shown). Immunoblot analysis of PKC isoforms in subcellular fractions was performed on the basal portions as described previously.[31] Briefly, samples were pulverized in homogenization buffer (20 mM Tris-HCl (pH 7.4), 2 mM ethylenediamine tetra-acetic acid, 0.5 mM ethylene glycol tetra-acetic acid, 100 nM aprotinin, 1 mM benzamidine, 1 μM leupeptin, 1 μM pepstatin, 1 mM phenylmethylsulphonyl fluoride) and centrifuged (100,000 *g* for 45 min, 4°C). The supernatant was saved as the cytosolic fraction, and the pellet was resuspended in homogenization buffer and saved as the membrane fraction.

Quantitative confocal immunofluorescence microscopy

Hearts were removed from the perfusion apparatus, fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned for immunohistochemistry and quantitative confocal microscopy as described previously.[32,33] The amount of Cx43 signal at intercellular junctions was quantified as described previously[32,33] and expressed as a proportion of total tissue area. The amount of phospho-Cx43(Ser368) at junctions was also quantified and expressed as a fraction of total Cx43 signal.

Measurement of whole-tissue resistance

Electrical uncoupling during ischemia was monitored in isolated mouse hearts by measuring changes in whole tissue resistance using the 4-electrode method.[7,34–37] Once perfusion with normoxic buffer had been initiated in excised hearts, 4 Teflon-coated silver wire electrodes (0.0015-inch 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