First molecular evidence that inositol trisphosphate signaling contributes to infarct size reduction with preconditioning

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Przyklenk, Karin, Michelle Maynard, and Peter Whittaker. First molecular evidence that inositol trisphosphate signaling contributes to infarct size reduction with preconditioning. Am J Physiol Heart Circ Physiol 291: H2008–H2012, 2006. —Considerable attention has focused on the role of protein kinase C (PKC) in triggering the profound infarct-sparing effect of ischemic preconditioning (PC). In contrast, the involvement of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃], the second messenger generated in parallel with the diacylglycerol-PKC pathway, remains poorly understood. We hypothesized that, if Ins(1,4,5)P₃ signaling [i.e., release of Ins(1,4,5)P₃ and subsequent binding to Ins(1,4,5)P₃ receptors] contributes to PC-induced cardioprotection, then the reduction of infarct size achieved with PC would be attenuated in mice that are deficient in Ins(1,4,5)P₃ receptor protein. To test this concept, hearts were harvested from 1) B6C3Fe-ala-Itpr-1 opt/− mutants displaying reduced expression of Ins(1,4,5)P₃ receptor-1 protein, 2) Itpr-1 opt/−/− wild types from the colony, and 3) C57BL/6J mice. All hearts were buffer-perfused and randomized to receive two 5-min episodes of PC ischemia, pretreatment with d-myo-Ins(1,4,5)P₃ [sodium salt of native Ins(1,4,5)P₃], the mitochondrial ATP-sensitive K⁺ channel opener diazoxide, or no intervention (controls). After the treatment phase, all hearts underwent 30-min global ischemia followed by 2 h of reperfusion, and infarct size was delineated by tetrazolium staining. In both wild-type and C57BL/6J cohorts, area of necrosis in hearts that received PC, d-myo-Ins(1,4,5)P₃, and diazoxide averaged 28–35% of the total left ventricle (LV), significantly smaller than the values of 52–53% seen in controls (P < 0.05). In contrast, in Itpr-1 opt/−/− mutants, protection was only seen with diazoxide: neither PC nor d-myo-Ins(1,4,5)P₃ limited infarct size (52–58% vs. 56% of the LV in mutant controls). These data provide novel evidence that Ins(1,4,5)P₃ signaling contributes to infarct size reduction with PC.

myocardial infarction; signal transduction; inositol 1,4,5-trisphosphate

PRECONDITIONING (PC) is the well-described phenomenon whereby brief episodes of myocardial ischemia render the heart resistant to a subsequent, sustained ischemic insult (21, 29). Among the multiple, complex mechanisms that purportedly contribute to the profound infarct-sparing effect of PC, considerable attention has focused on the pivotal role of protein kinase C (PKC) in triggering the preconditioned state (6, 29). In contrast, the involvement of inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃], the second messenger generated in parallel with the diacylglycerol-PKC pathway, remains poorly understood. Specifically, while preischemic administration of exogenous Ins(1,4,5)P₃ [i.e., d-myo-Ins(1,4,5)P₃ hexadecanol] has been shown to evoke a reduction of infarct size comparable in magnitude to that achieved with PC (9, 22, 23), and indirect evidence has implicated the possible involvement of native Ins(1,4,5)P₃ in PC- and opioid-induced cardioprotection (1, 2), efforts to gain definitive insight into this issue have been confounded by the lack of selective and short-acting Ins(1,4,5)P₃ antagonists (4). We hypothesized that, if Ins(1,4,5)P₃ signaling [that is, release of Ins(1,4,5)P₃ and subsequent binding to Ins(1,4,5)P₃ receptors] contributes to infarct size reduction with PC, then the infarct-sparing effect of PC would be attenuated in mice that are deficient in Ins(1,4,5)P₃ receptor protein. Accordingly, our goal was to test this concept in mice displaying a defect in the Ins(1,4,5)P₃ receptor-1 gene (Itpr-1 opt).

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and was performed in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animals Resources (NIH Publication Vol. 25, No. 28, Revised 1996).

Experiments were conducted using adult (3–4 mo old) B6C3Fe-ala-Itpr-1 opt/−/− mice heterozygous for the opisthotonus spontaneous mutation (purchased from The Jackson Laboratory, Bar Harbor, ME). Mutant Itpr-1 opt/−/− mice are reportedly characterized by a reduced expression of Ins(1,4,5)P₃ receptor-1 protein and loss of modulatory sites (26). Of note, heterozygous Itpr-1 opt/−/− mice were utilized, as animals homozygous for the spontaneous mutation die soon after birth (16). Concurrent experiments were performed in two age-matched control cohorts: wild-type Itpr-1 opt/−/− mice from the colony (The Jackson Laboratory) and a standard population of C57BL/6J mice.

Characterization of Itpr-1 opt/−/− mice. To gain initial insight into the phenotype of the Itpr-1 opt/−/− mutants, mice from each of the three cohorts were anesthetized with pentobarbital sodium (60 mg/kg ip) and 1) the hearts and brains were rapidly excised, snap-frozen in liquid nitrogen, and used for detection (by standard immunoblotting) of Ins(1,4,5)P₃ receptor-1 protein (n = 3 per cohort); or 2) the hearts were harvested for histological assessment of myocyte size and myocardial fibrosis (n = 4).

Infarct size protocol. Myocardial infarct size, our primary study endpoint, was assessed using the isolated buffer-perfused heart model. In brief, Itpr-1 opt/−/− mutants (n = 24), Itpr-1 opt/−/− wild types (n = 24), and C57BL/6J mice (n = 32) were anesthetized with pentobarbital, and the hearts were rapidly excised and immediately mounted on an aortic cannula for retrograde perfusion (nonrecirculating) at a constant pressure of 55 mmHg. The buffer was composed of (in mM) 118 NaCl, 4.7 KCl, 24 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 11 glucose, and 2.5 CaCl₂ anhydrous in distilled water at a pH of 7.4, and was continuously oxygenated with 95% O₂-5% CO₂. Care was taken to maintain both buffer temperature and heart temperature at 37°C. A balloon constructed of polyvinylchloride plastic film was inserted into...
the left ventricle (LV), inflated to an end-diastolic pressure of 5 mmHg, and used to monitor cardiodynamic function throughout the experiment.

After stabilization, all hearts underwent a 25-min intervention phase (Fig. 1). For each of the three cohorts, hearts were assigned to receive uninterrupted buffer perfusion (controls), brief PC ischemia, or exogenous administration of d-myo-Ins(1,4,5)P₃ hexasodium (Calbiochem, La Jolla, CA). The PC stimulus consisted of two 5-min episodes of global ischemia interspersed with 5 min of reflow and followed by 10 min of reperfusion, while d-myo-Ins(1,4,5)P₃ was dissolved in buffer and given over 1 min via a side port located immediately proximal to the heart at a final concentration (6 μM) shown previously to limit infarct size (9, 22, 23). In addition, to determine whether reduction of infarct size could be evoked by triggering a more distal signaling component common to both PC- and d-myo-Ins(1,4,5)P₃-induced cardioprotection [in particular, opening of mitochondrial ATP-sensitive K⁺ (KATP) channels (8, 23, 29)] hearts in each cohort received 15 min of standard buffer perfusion followed by a 10 min infusion of diazoxide [Sigma, St. Louis, MO; final concentration in buffer: 100 μM (18)].

After the 25-min intervention period, all hearts underwent 30 min of sustained global ischemia followed by 2 h of reperfusion (Fig. 1). The hearts were then cut into four to six transverse slices, and the extent of necrosis was delineated by triphenyltetrazolium staining (incubation for 15 min at 37°C). All hearts were digitally photographed, and infarct size was quantified in a blinded manner (without knowledge of the treatment group) by using image analysis software (SigmaScan Pro: Systat, Point Richmond, CA).

For each of the three cohorts, infarct size (expressed as a % of total LV weight) in control, PC, d-myo-Ins(1,4,5)P₃, and diazoxide-treated groups was compared by ANOVA, and, if significant F values were obtained, pairwise comparisons were made using the Student-Newman-Keuls test.

RESULTS

We confirmed that, as expected, mutant Ipr-1opt-/+ mouse displayed a ~50% deficit in Ins(1,4,5)P₃ receptor-1 protein expression when compared with wild-type Ipr-1opt+/+ or C57BL/6J mice (Fig. 2). However, there were no differences in myocyte size, myocardial fibrosis, heart weight-to-body weight ratios, or baseline cardiodynamic performance (heart rate, LV developed pressure, dP/dt) among the three cohorts. In addition, LV function after relief of sustained global ischemia recovered to 30–40% of baseline values in all hearts, irrespective of the strain or the treatment group (data not shown).

For C57BL/6J mice, infarct size in control hearts averaged 53% of the total LV. Moreover, area of necrosis was, as expected, reduced to 30–34% of the LV in PC-, d-myo-Ins(1,4,5)P₃-, and diazoxide-treated groups vs. controls (Figs. 3 and 4). Similar results [i.e., significant cardioprotection with PC, d-myo-Ins(1,4,5)P₃, and diazoxide] were observed in hearts from wild-type Ipr-1opt+/+ mice (Fig. 4). In contrast, in the Ipr-1opt-/+ cohort, only diazoxide-treated hearts displayed a significant reduction of infarct size when compared with matched controls: neither d-myo-Ins(1,4,5)P₃ nor PC evoked an infarct-sparing effect in Ins(1,4,5)P₃ receptor-1-mutant mice (Figs. 3 and 4). To rule out the concept that two 5-min episodes of PC ischemia may have constituted a subthreshold stimulus, two additional Ins(1,4,5)P₃ receptor-1-mutant mice were enrolled in a post hoc manner and received four (rather than two) 5-min cycles of PC ischemia. The amplified PC stimulus was, similarly, ineffective in initiating protection: i.e., infarct size in these supplemental hearts averaged 63% of the total LV.

DISCUSSION

The established, classical role of Ins(1,4,5)P₃ lies in the control of calcium homeostasis (specifically, mobilizing the release of calcium from intracellular stores), initiated by binding of the second messenger to members of the Ins(1,4,5)P₃ receptor family (3). However, in cardiomyocytes, in which ryanodine receptors serve as the primary calcium release channel for excitation-contraction coupling, the purpose of Ins(1,4,5)P₃ is unclear (15, 27). Indeed, while the requisite components of Ins(1,4,5)P₃ signaling [including Ins(1,4,5)P₃ receptor-1-, -2, and -3 subtypes] are present in the myocardium (10, 13, 20, 27), the roles (s) of Ins(1,4,5)P₃ signaling in the heart remain poorly defined. In this regard, the current results obtained in Ipr-1opt-/+ mice provide novel evidence that Ins(1,4,5)P₃ signaling contributes to the reduction of infarct size seen with ischemic PC.
Our data imply the specific involvement of the Ins(1,4,5)P₃ receptor-1 subtype in the infarct-sparing effect initiated by both PC and exogenous D-myocardial Ins(1,4,5)P₃. These data are perhaps surprising, given the relative proportion and distribution of type-1 versus type-2 Ins(1,4,5)P₃ receptors in the heart. In LV homogenates, the abundance of type-1 and type-2 receptors is approximately equal. However, in isolated cardiomyocytes, the predominant isoform is undoubtedly the type-2 Ins(1,4,5)P₃ receptor, with neonatal myocytes purportedly expressing only the receptor-2 subtype (5, 7, 10, 15, 17). This disparity between preparations may reflect an abundance of type-1 receptor on nonmyocytes (i.e., endothelial cells), a concept that is problematic as it is unclear how stimulation of Ins(1,4,5)P₃ receptors on nonmyocytes would result in myocardial salvage. Expression of the receptor-1 subtype has, however, been detected in isolated adult cardiomyocytes by immunohistochemistry and in situ hybridization (5, 15, 17). Moreover, it is possible that low expression of type-1 Ins(1,4,5)P₃ receptors in cardiomyocytes may be due in part to a loss of receptors, in particular, the population reportedly localized at intercalated...
discs (12), during dissociation and myocyte isolation. This concept, albeit speculative, may be noteworthy, as previous evidence from our group suggests that cardioprotection achieved with d-myo-Ins(1,4,5)P₃ is triggered via stimulation of Ins(1,4,5)P₃ receptors localized in proximity to gap junctions/hemichannels (22).

A second intriguing aspect of this study is that, in heterozygous Ins(1,4,5)P₃ receptor-1-mutant mice displaying a partial deficit in Ins(1,4,5)P₃ receptor-1 protein, there was no evidence of partial, intermediate, or incomplete cardioprotection with PC. Rather, infarct sizes in hearts that received PC ischemia were comparable to those seen in matched controls. This concept is not without precedent: similar results (that is, a complete failure of PC to limit infarct size) has been observed in heterozygous mice exhibiting a 50% deficiency in the gap junction protein, connexin 43 (24, 25). We cannot, however, speculate on the minimum threshold deficit in Ins(1,4,5)P₃ receptor protein-1 expression that results in a total loss of infarct size reduction with PC.

The current data showing an absence of PC-induced cardioprotection in Itpr-1opt+/− mutants suggest that the Ins(1,4,5)P₃ receptor-1 subtype is an integral component of (i.e., in series with, rather than in parallel with or alternative to) the signaling pathway responsible for PC. However, it is unclear whether the inability of PC [and exogenous d-myo-Ins(1,4,5)P₃] to trigger protection in the mutant cohort is due entirely to the deficit in Ins(1,4,5)P₃ receptor-1 protein or whether Itpr-1opt+/− mice may display more global (and as-yet uncharacterized) defects in cardioprotective signaling. Although definitive resolution of this issue is beyond the scope of the current study, two prospectively focused on a signaling element common to both this issue is beyond the scope of the current study, two


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