Mitochondria and Preconditioning
A Connexin Connection?

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In recent years it has become widely accepted that mitochondria play a critical role in determining whether or not a heart recovers on reperfusion after a period of ischemia. A critical process in initiating cell death is the opening of the mitochondrial permeability transition pore (MPTP), a nonspecific pore in the inner mitochondrial membrane (IMM), that causes mitochondrial uncoupling and swelling. MPTP opening is triggered by matrix calcium overload, especially when accompanied by oxidative stress and adenine nucleotide depletion, conditions known to occur during ischemia. During ischemia MPTP opening is inhibited by low pH, but on reperfusion opening only occurs when the pH returns to normal accompanied by a further burst of reactive oxygen species (ROS). Myocytes cannot maintain their ATP levels if the pores remain open leading to disruption of ionic homeostasis and ultimately necrosis, whereas transient pore opening may initiate apoptosis through MPTP-induced release of factors such as cytochrome C that activate the apoptotic caspase cascade. The importance of the mitochondrial pathway in mediating reperfusion injury has been confirmed through the use of specific MPTP inhibitors such as cyclosporin A (CsA) and sanglifehrin A (SFA) that afford protection against reperfusion injury, as does genetic knock-out of Cyp-D, a key component of the MPTP.

Ischemic preconditioning (IPC) offers potent protection against reperfusion injury and has been shown to involve inhibition of MPTP opening, although the exact mechanisms responsible remain unclear. Our own data imply an indirect effect, probably mediated by a decrease in ROS, while others have championed a wide variety of more direct effects on mitochondria involving various protein kinases. There is also an ongoing debate as to the role of \( K_{\text{ATP}} \) channels in preconditioning and especially those proposed to reside in the mitochondrial inner membrane. However, the extensive literature reveals no consensus as to how all these kinase pathways and potassium channels might interact to inhibit the MPTP and so mediate protection. Recently, another contender that might connect preconditioning and the mitochondria has been proposed: connexin43 (Cx43). This is the focus of the paper by Rodriguez-Sinovas et al in this issue of Circulation Research. Cx43 is the major gap junction protein with hexameric assemblies forming connexons on adjacent cardiomyocytes. These link the cells, providing a route for intercellular communication through the propagation of action potential, signaling molecules, and metabolites. Hexameric assemblies of Cx43 occurring elsewhere on the sarcolemma (hemichannels) may play a role in volume regulation. The permeability of connexons and hemichannels is regulated through several mechanisms including \( [\text{Ca}^{2+}] \), pH, and phosphorylation by several protein kinases, including PKCe and PKG, both of which have been implicated in IPC. Cx43 is normally partially phosphorylated with low conductance, but progressive dephosphorylation occurs during ischemia causing increased conductance. This may be important in propagating injury from one cell to another, because some studies have shown protection of hearts from ischemia–reperfusion injury when gap junctions are uncoupled with heptanol. However, previous work from the group of Heusch and Schulz demonstrated that hearts from Cx43-deficient mice were not protected from reperfusion injury, but they could not be preconditioned, leading the authors to conclude that Cx43 might play a critical role in IPC. Subsequent studies in pig and rat hearts confirmed that the dephosphorylation of Cx43 and electrical uncoupling during ischemia was prevented by IPC in a PKC-dependent manner, and that IPC increased the colocalization of several protein kinases with Cx43 during ischemia. Although these data are consistent with a role for Cx43 in preconditioning, they offer few clues as to the mechanisms involved. Indeed, one might anticipate that if phosphorylation of Cx43 induces connexon closure, then the Cx43-deficient mice should have lower gap junction conductance after ischemia–reperfusion and hence exhibit protection. Because this is not the case, another mechanism of Cx43 action is implied. Might this involve mitochondria?

Last year the groups of Garcia-Dorado, Di Lisa, Heusch, and Schulz reported that a small fraction of Cx43, primarily in its phosphorylated form, was present in purified mitochondria from rat, mouse, pig, and human hearts, and that its presence increased after IPC. The authors used a wide range of different techniques to eliminate the possibility that these data represent an artifact mitochondrial contamination by the sarcolemma. Western blots of mitochondrial fractions with sarcolemmal markers, immunofluorescence colocalization studies of Cx43 with mitoTracker red or cytochrome C, and immunogold electron microscopy of cardiac myocyte sections all confirmed a mitochondrial localization for Cx43, and we have been able to confirm these data in our own laboratory (Clarke S, unpublished data, 2006). So what is the role for mitochondrial Cx43? In another series of experiments, the authors demonstrated that hearts from Cx43-deficient mice...
could not be preconditioned with diazoxide. This agent has been used as a specific mitochondrial K\textsubscript{ATP} channel opener, although its actual mode of action is less clear and may include nonspecific effects on mitochondria, including inhibition of succinate dehydrogenase and uncoupling. Nevertheless, irrespective of its target, diazoxide causes the formation of ROS, an established signal for preconditioning, and this was inhibited in Cx43-deficient hearts that also lacked diazoxide-mediated protection. The authors concluded that recruitment of Cx43 to the mitochondria in IPC might play a role in the production of ROS that mediates preconditioning, although the mechanism remained unclear.

Another weak link in the authors’ hypothesis is the location of mitochondrial Cx43. Although their earlier data had suggested that Cx43 associates with the cristae, these data might represent a fixation artifact. They do not totally exclude the possibility that Cx43 remains tightly bound to the external surface of the mitochondria rather than translocated into the matrix or IMM. In their article published in this issue of Circulation Research they address this issue by using mitochondrial subfractionation techniques. They demonstrate that removal of the majority of the outer mitochondrial membrane (OMM) by digitonin treatment did not reduce the amount of Cx43 in the mitochondria relative to the adenine nucleotide translocase (ANT, an IMM protein), yet greatly reduced the amount of voltage dependent anion channel (VDAC, an OMM protein) as revealed by both immunofluorescence confocal microscopy and Western blotting. Additional fractionation procedures yielded various matrix, IMM, and OMM fractions, and again Cx43 most closely followed the ANT distribution rather than matrix, OMM, or intermembrane space markers. This led the authors to conclude that Cx43 is translocated to the IMM, although some Cx43 may remain associated with the OMM.

They extended their studies by investigating the mechanism of Cx43 translocation into the mitochondria. Protein translocation across the OMM involves the TOM complex, either alone or in cooperation with other translocation proteins. Proteins destined for the matrix or IMM use additional inner membrane translocation complexes, TIM23 and TIM22, respectively. In many cases, recognition by the TOM complex involves an N-terminal targeting presequence, although proteins lacking this sequence can use internal targeting sequences but must first interact with the cytosolic chaperones Hsp70 and Hsp90 to be recognized by TOM22. Because Cx43 lacks an N-terminal presequence it must use the latter mechanism, and so the authors used coimmunoprecipitation experiments to reveal any interaction between Cx43 and TOM20 or Hsp90. They demonstrated the presence of both Hsp90 and TOM20 in a Cx43 immunoprecipitate and confirmed the interaction by showing that Cx43 coimmunoprecipitated with TOM20 and Hsp90 antibodies. Although these data may appear convincing, coimmunoprecipitation experiments are notoriously prone to false-positive results because transmembrane helical domains of membrane proteins are very hydrophobic and tend to associate nonspecifically with other membrane proteins even in detergent solution. Some indication that this may be the case in the authors’ experiments is the significant amount of ANT found in the Cx43 immunoprecipitate. To provide more convincing data for the localization of Cx43 within mitochondria, protection of mitochondrial Cx43 from proteolytic cleavage might be the method of choice.

If it is assumed that Cx43 is translocated into the mitochondria during preconditioning, is this important for mediating the protection? To answer this question the authors looked at the effect of geldanamycin, a blocker of Hsp90-dependent translocation through the TOM pathway. They were able to demonstrate that 15 minutes treatment of hearts with this agent reduced the amount of Cx43 in mitochondria and prevented the IPC-induced increase in mitochondrial Cx43. However, this inhibition of translocation was not accompanied by a loss of protection determined using hemodynamic function, LDH release, and infarct size. By contrast, neither diazoxide nor isoprenaline induced translocation of Cx43 to the mitochondria, yet in both cases protection was blocked or reduced by geldanamycin. Surely, these data indicate that Cx43 association with mitochondria has no relevance to preconditioning? However, the authors argue otherwise and invoke “redundancy in signaling pathways.” They propose that Cx43 is involved in the production of ROS by mitochondria in some ill-defined way that requires a threshold concentration of Cx43. Cx43-deficient mice show a drop in mitochondrial Cx43 below this threshold and so cannot produce a preconditioning ROS signal, whereas geldanamycin does not reduce mitochondrial Cx43 sufficiently to exert such an effect. But then why does it prevent diazoxide preconditioning? The authors suggest that the Cx43 is especially important in the mitoK\textsubscript{ATP} channel–mediated mechanism of preconditioning, but the exact role of this pathway is extremely unclear and may not even exist.
What are we to conclude? There seems little doubt that Cx43 can associate with mitochondria after IPC, but the evidence that it is translocated into the matrix or IMM rather than bound to the outside is less convincing. Furthermore, the loss of preconditioning in Cx43-deficient hearts argues strongly for some pool of Cx43 playing a critical role, and because mitochondria are an end target for cardioprotection, there must be some interaction, direct or indirect, between this Cx43 and mitochondria. The nature of this link remains mere speculation, although the authors make a strong argument that ROS may play a key role. The scheme in the Figure tries to reconcile these proposals with some of the critical points raised above.

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**References**

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