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Review

Studying ischemic preconditioning in isolated cardiomyocyte models

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Abstract

Isolated cardiomyocytes, obtained by enzymatic digestion of whole hearts, have multiple advantages, most related to their accessibility to microscopic visualization, beyond the obvious elimination of other cell types that exist in the heart. Conversely, they cannot reproduce the mechanical disruption of reperfusion hypercontracture or the vascular phenomena of leukocyte plugging and compression from interstitial edema and contracture that can lead to the no-reflow phenomenon. Nevertheless, ischemic preconditioning has been consistently demonstrated to be a potent protective mechanism in freshly isolated and cultured cardiomyocytes across multiple species, indicating that much of the innate protection of ischemic preconditioning resides in cardiomyocytes. Centrifuging freshly isolated cardiomyocytes into a pellet with only a thin layer of supernatant covered by oil has proven to be an excellent model of simulated ischemia. In culture, cardiomyocytes may be exposed to severe hypoxia only or to various protocols for simulated ischemia in which an acid/lactate-rich, hyperkalemic extracellular environment with substrate deprivation (lacking glucose) is typically added. Reperfusion is simulated by well-oxygenated media of normal ionic composition. Cardiomyocyte injury has been usually evaluated by cell membrane permeability to dyes, often under hypo-osmotic conditions (osmotic fragility) or enzyme release. A survey of the use of cardiomyocyte models to study preconditioning is presented with the emphasis on examples of the innovative measurements, increasingly involving molecular techniques, that point to an increasing future role for these models in preconditioning research and, more generally, in the mechanistic study of myocardial ischemia/reperfusion.

Keywords: Ischemic preconditioning; Simulated ischemia/reperfusion; Cardiomyocytes; Cell culture

1. Introduction

Ischemic preconditioning (IPC), first discovered by Murry et al. [1], is classically defined as a phenomenon by which one or more cycles of brief (5 to 10 min) myocardial ischemia followed by also brief period(s) of reperfusion render the heart (myocardium) protected against a subsequent prolonged (index) period of myocardial ischemia and reperfusion. This relatively immediate protection known as "classical ischemic preconditioning" is known to dissipate within 1 to 2 h following the initial IPC stimulus [2]. Protection reappears from about 24 to 72 h after the initial preconditioning stimulus, and has been termed "late ischemic preconditioning" or "second window of protection" of ischemic preconditioning [3].

This review concerns the use of isolated adult cardiomyocytes from a variety of species and developmental stages (embryonic, neonatal and mature adult) as a research tool to gain insight into how ischemic preconditioning works to achieve cardioprotection. Given limitations of space, it cannot be comprehensive regarding the current knowledge of mechanisms of preconditioning nor even in relation to all of the uses of cardiomyocytes to study preconditioning. Our limited focus is on the approaches used to simulate ischemia and reperfusion in isolated cardiomyocytes, the techniques to assess cellular injury, and both the advantages and disadvantages inherent in using

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isolated cardiomyocytes to test hypotheses concerning preconditioning. We present some examples in which aspects of myocardial preconditioning could not be studied without resorting to isolated cardiomyocytes, to emphasize the utility of this approach. This review does not include patch-clamp or related electrophysiological studies on single cardiomyocytes nor the use of cardiomyocytes to study delayed preconditioning.

2. A brief review of ischemic preconditioning mechanisms

It is well established that preconditioning is triggered by substances released from tissue during brief periods of ischemia and reperfusion and the tissue need not even be myocardium as indicated by the cardioprotection realized through "remote preconditioning" involving, for example, tourniquet occlusions of the vascular beds of skeletal muscles in the same individual or brief occlusion and reflow of a mesenteric artery [4]. Specific receptors on the cardiomyocyte cell membrane, including but not necessarily adenosine A_1 and A_3, bradykinin B_2, α_1-adrenergic, AT1 angiotensin II, muscarinic M_2, and δ-opioid receptors, are stimulated by these substances [5]. The downstream signaling cascades from these receptors are complex and a detailed description is beyond the terms of reference for this review. However, it is clear that multiple protein kinases are involved, notably one or more isoforms of protein kinase C [6–8] and at least two tyrosine kinases (e.g., Src and PI3 kinase) [9]. There is good evidence that multiple, redundant signaling pathways lead to cardioprotection [10]. While it is relatively easy to block single cycle ischemic preconditioning by a variety of interventions, multi-cycle preconditioning results in more potent protection, as we demonstrated in vivo [11], which is more difficult to block. Thus, while preconditioning is a threshold phenomenon, its protection is not as simple as all-or-none in the heart in vivo. Simultaneous pharmacological blockade by both protein kinase C and tyrosine kinase activities have been demonstrated to be necessary to abolish the cardioprotection of multi-cycle ischemic preconditioning in rats [12,13] and even single cycle ischemic preconditioning in the pig [14]. The involvement of these kinases suggests that the "memory" of classic ischemic preconditioning is embodied in the phosphorylation state of likely multiple end effector proteins which through the consequences of their level of activity achieve cardioprotection, specifically the prolongation of the long (index) ischemic interval which results in a specific amount of cardiomyocyte cell death.

Candidates for end effectors in ischemic preconditioning include channel proteins in the cardiomyocyte cell membrane, with the sarcolemmal K_{ATP} channel [15–17], Cl^- channels [18–20] including the CFTR channel [21], the inward rectifier K^+ channel I_{K1} [22] and connexin 43 related channels [23] as contenders. In the mitochondria, the mitochondrial K_{ATP} channel [24] and the mitochondrial permeability transition pore [25,26] are candidates. Establishing the end effectors of ischemic preconditioning will be essential to truly understand this fascinating phenomenon. It would seem intuitive that the study of isolated cardiomyocytes should be able to contribute valuable insights into the mechanistic understanding of preconditioning, which cannot be achieved by a study limited to the whole heart, in vivo or in vitro.

3. Advantages of isolated cardiomyocytes in studying ischemic preconditioning

Perhaps the most obvious advantage of studying cardiomyocytes compared to the whole heart is the elimination of other cell types, notably endothelial cells and fibroblasts, so that their influence is negligible. For example, in studying the cell signaling involved in preconditioning to subcellular components such as the cell membrane versus mitochondria, it is desirable to have a pure collection of cardiomyocytes rather than the various cell types present in the whole heart. Most of the advantages of cardiomyocytes flow from the ability to visualize them; microscopy invariably plays a key role in cardiomyocyte studies. Through repeated visualization of the cells it is possible to use the cardiomyocyte as its own control. However, the ability to repeatedly visualize the cell is attended by significant limitations, most obviously that each cardiomyocyte must be identifiable to allow a return to visualize that cell. This requires the cardiomyocytes studied to be stationary which translates in practice into adhering them to a plate in primary cell culture. The alternative is to collect observations from different samples of cardiomyocytes. This may work but can be problematic if there is substantial cell-to-cell variability for the parameter under study as we have experienced for example with cell volume due to the substantial normal variability in cardiomyocyte size. Thus, cardiomyocytes may be studied either when freshly isolated, with the limitation that repeated observations cannot be made on the same cell, or in culture without this limitation. Cell culture offers the additional advantage that targeted manipulation of gene expression can be achieved and a variety of imaging techniques can be applied. However, the simulation of ischemia is more challenging in cell culture, as explained below. Also, only a small fraction of cardiomyocytes from a heart is available on culture plates as compared with the fresh isolate. This may be a practical limitation for immunoblotting and immunoprecipitation assays. A much more abundant, but nevertheless pure, sample of cardiomyocytes is available from the fresh isolate. In practice, one can readily combine fresh isolates and a component plated for primary culture from the same heart.

4. Limitations of isolated cardiomyocytes in studying ischemic preconditioning

In separating cardiomyocytes from their syncytial neighbors and also from the blood vessels and surrounding extracellular matrix, important aspects of the pathophysiology of myocardial ischemia/reperfusion injury in the whole heart are lost. Isolated cardiomyocytes are not capable of reproducing the mechanics of reperfusion hyper-contracture in the whole heart so well described by Piper et al. [27,28] in which the cardiomyocytes exert so much force on each other that there are cytoskeletal and sarcolemmal disruptions which cause massive enzyme release and massive secondary influx of calcium ions into the broken cells. Furthermore, the endothelial lining of blood vessels subjected to ischemia/reperfusion becomes more permeable causing interstitial edema on reperfusion. Vascular plugging can also occur by promotion of adherence, activation and accumulation of neutrophils and monocytes in the myocardium subjected to ischemia/reperfusion. The combination of contracture, interstitial edema and vascular plugging by platelets, leukocytes and fibrin can lead to the no-reflow phenomenon. Study of isolated cardiomyocytes cannot address these phenomena. Nevertheless, ischemic protection was demonstrated to be a potent protective mechanism in the pioneering studies of Armstrong et al. [29,30] in freshly isolated cardiomyocytes and Ikonomidis et al. [31] in cultured cardiomyocytes. Experiments in isolated cardiomyocytes have generally reproduced the results on cardiomyocyte cell death in whole hearts of a wide variety of interventions exploring cellular mechanisms in preconditioning.

5. Studying ischemic preconditioning in freshly isolated cardiomyocytes

The use of isolated cardiomyocytes for studies in ischemic preconditioning can be traced back to the seminal work, published in 1990, of Vander Heide et al. [32], who developed an in vitro model of myocardial ischemia using freshly isolated rat cardiomyocytes. The separation of cardiomyocytes by enzymatic digestion of the myocardium dates back a decade earlier to a work by Altschuld et al. [33,34]. The enzymatic separation of cardiomyocytes can be done by a variety of protocols, the key elements of which are Langendorff perfusion of an isolated heart by an almost calcium-free medium containing collagenase and trypsin. Nominally calcium-free media can contain about 50 μM calcium so that EGTA is used to reduce this further [35]. This is a delicate procedure with the yield of viable cardiomyocytes dropping with too little or too much collagenase or trypsin activity (to be distinguished from simple concentration) and too short or too long a duration of perfusion. Using this approach, we have been able to obtain 20 to 30 million cardiomyocytes of which 70% to 80% or more show rod-shaped morphology and readily visible striations under the microscope. We have found that adding Ca^{2+} back to a concentration of about 1 mM is best done in four or five small increments over a period of about 30 min. More information on cardiomyocyte isolation is posted on the H.E.L.P files of the International Society for Heart Research website (www.ishrworld.org).

Armstrong, working with Ganote, was first to study ischemic preconditioning in isolated cardiomyocytes [29]. They used the Vander Heide et al. [32] in vitro simulated ischemic model in which freshly isolated cardiomyocytes were centrifuged at a very slow speed into a pellet (about 0.25 ml, 0.8 to 1 cm thick) in a microcentrifuge tube with excess supernatant removed so that the fluid layer over the cells was limited to about one-third the volume of the pellet. The supernatant was covered with an oil layer and incubated without agitation at 37 °C. Armstrong was able to precondition by either incubation in the absence of glucose [30] or by brief simulated ischemia by pelleting [29]. Using this simulated ischemia model, these investigators assessed the level of ischemic injury by determining the percentage of cardiomyocytes that were stained with trypan blue after resuspension of samples obtained from inside the ischemic pellet, at different time points, in a hypotonic (85 mOsm) modified Tyrode solution with reduced NaCl containing 0.5% glutaraldehyde, 3 mM amytal and 1.0% trypan blue. Trypan blue, a 961 kDa dye, will enter cardiomyocytes through a damaged cell membrane, staining the entire cardiomyocyte and indicating the cell to be irreversibly injured [34]. Simultaneously, in the same cardiomyocytes, Armstrong and Ganote [29] measured the percentage of cardiomyocytes becoming progressively squared (cardiomyocytes in which the length : width ratio was transformed from 6 : 1 to near 1 : 1) over time during simulated ischemia, indicating the presence of ischemic contracture. In fact, the increase in the percentage of squared cardiomyocytes in their ischemic pellet was associated with a parallel decrease in ATP, increase in lactate and increase in cell death. Based on the measurement of the percentage of blue (dead) cardiomyocytes measured at different time points during 180 min of simulated ischemia, it was possible for them to construct mortality curves over that time period of simulated ischemia and compare the integrated areas under the mortality curves.

Evaluating cardiomyocyte viability by trypan blue staining has become the gold standard in ischemic preconditioning studies in either freshly isolated or cultured cardiomyocytes [18,22,30,36–39]. The hypotonicity of the staining solution used by Armstrong and Ganote [29] places an additional mechanical stress on the damaged, osmotically fragile cell membranes so that cardiomyocytes of borderline membrane permeability swell and rupture. Amytal, a reversible inhibitor of mitochondrial complex I (NADH–ubiquinone–oxidoreductase) which blocks mitochondrial ATP production on reoxygenation, was used to reduce contracture. The focus of these pioneering studies by

Armstrong and Ganote [29] was on evaluating cell viability, in effect osmotic fragility, after a few minutes at 85 mOsm, in 25 μL aliquots of cardiomyocytes obtained from the pellet simulating ischemia, not on simulating reperfusion.

Our innovation was to simulate prolonged (60 min) reperfusion in the simulated ischemia pelleting model of Vander Heide et al. [32]. We resuspended cardiomyocytes from the pellet in an *iso*-osmotic physiological solution following 30, 45, 60 and 90 min of simulated ischemia evaluating their viability in *iso*-osmotic trypan blue at the end of 60 min of simulated reperfusion, and also examined ultrastructural changes comparing ischemic preconditioning (by 10 min pelleting followed by 10 min of simulated reperfusion immediately prior to the pelleting index simulated ischemia) with non-preconditioned controls. We found that on electron microscopy of cardiomyocytes removed immediately after 30, 45 and 60 min of index ischemia simulated by pelleting, prior to simulated reperfusion, that ischemic preconditioning delayed the appearance of ultrastructural signs of irreversible injury and this was also reflected in a reduction of cell mortality as assessed by hypotonic trypan blue staining of these samples [40]. We compared our simulated ischemia/simulated reperfusion protocol using 45 min for the index simulated ischemia with Armstrong's ischemia only protocol and found improved cardiomyocyte survival with preconditioning but different shapes of the mortality curves [18]. Several research groups have studied preconditioning using freshly isolated cardiomyocytes as summarized in Table 1. Most have used pelleting under oil to simulate ischemia. We believe the pelleting model mimics ischemia with the greatest fidelity as it combines severe hypoxia with gradually increasing metabolite build-up in an enclosed space with metabolic inhibition occurring naturally through

Table 1
Freshly isolated cardiomyocyte models applied to ischemic preconditioning studies

References	Cardiomyocyte model	IPC stimulus	Simulated ischemia (SI) model	Method of assessing cell injury	Endpoint studied
[41]	Isolated rat cardiomyocytes	Not applicable	Ischemic pelleting. Glucose-free medium	Trypan blue staining and ultrastructure	Contracture, cell viability and osmotic fragility
[29,30, 42–46]	Isolated rabbit cardiomyocytes	MI, brief SI or PPC	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability
[44]	Isolated pig and rabbit cardiomyocytes	Brief SI/SR	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability, ATP, lactate and contracture
[47]	Isolated rabbit ventricular myocytes	Brief SI	NaCN+glucose-free medium, pH 6.6, high K^+, lactate, electrical stimulation (1 Hz)	Hypercontracture	Contractile function
[48]	Isolated rabbit ventricular myocytes	PPC	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability and mitochondrial flavoprotein oxidation
[18,20]	Isolated rabbit ventricular myocytes	Brief SI/SR	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability, osmotic fragility, ischemic cell swelling and cell volume regulation
[49]	Isolated rabbit ventricular myocytes	Brief SI/SR	Ischemic pelleting	Trypan blue staining	Cell viability
[50]	Isolated rabbit ventricular myocytes	Brief SI/SR	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability and osmotic fragility
[39]	Isolated rat cardiomyocytes	Brief SI/SR	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability
[51]	Isolated rabbit ventricular myocytes	PPC	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability and osmotic fragility
[52]	Isolated guinea-pig ventricular myocytes	Brief SI/SR	Hypoxia (100% Argon) in the presence of electrical stimulation (0.5 Hz)	Hypercontracture and Ca^{2+} overload	Contractile function, cell morphology and $[Ca^{2+}]i$
[53]	Isolated STAT-3 KO mouse cardiomyocytes	Brief SI/SR (SI buffer pH at 6.4)	Hypoxia (94% N_2–1% O_2–5% CO_2)+glucose-free medium (pH=6.2)	Trypan blue and morphology	Cell viability
[54]	Isolated rat ventricular myocytes	Brief SI/SR	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability and cGMP levels
[55]	Isolated connexin 43 (+/−) mouse cardiomyocyte	Brief SI/SR	Ischemic pelleting	Hypo-osmotic trypan blue staining	Cell viability

SR = simulated reperfusion (reoxygenation); PPC = pharmacological preconditioning; MI = metabolic inhibition; IPC = preconditioning.

the build-up of lactate and H^+ and exhaustion of available metabolic substrates, avoiding the use of exogenous metabolic inhibitors.

6. Studying ischemic preconditioning in cultured cardiomyocytes

Cardiomyocytes in primary culture have been used by several research groups to study preconditioning as summarized in Table 2. Indeed, one of the earliest studies of ischemic preconditioning in isolated cardiomyocytes used human ventricular cardiomyocytes in primary culture [31]. A variety of techniques have been used to simulate ischemia in culture. Severe hypoxia, using nitrogen concentrations between 95% and 100%, has been used consistently. Substrate deprivation through the use of a glucose-free medium has been used by several groups (see Table 2) and we have used a variant on this theme, substituting non-metabolizable 2-deoxyglucose for glucose [22]. We preconditioned cultured cardiomyocytes by exposing them for 10 min to a hypoxic ($PO_2 < 8$ mm Hg) HEPES-based solution without substrate (glucose), with physiological concentration of KCl (4.5 mmol/L),

Table 2
Cultured cardiomyocyte models applied to ischemic preconditioning studies

References	Cardiac cell model	IPC stimulus	Simulated ischemia (SI) model	Method of assessing cell injury	Endpoint studied
[31, 56]	Human ventricular myocytes	Brief SI/SR or PPC	Anoxia (100% N_2)	Trypan blue staining+ LDH measurements	Enzyme release, cell viability, ATP and lactate
[57]	Chick embryo ventricular myocytes	Brief SI/SR	Hypoxia (100% N_2)	Trypan blue staining, creatine kinase and measurements	Enzyme release and cell viability
[58–61]	Chick embryo ventricular myocytes	Brief SI/SR or PPC	Hypoxia (100% N_2)	Trypan blue staining+ creatine kinase measurements	Enzyme release, cell viability and Ca^{2+} uptake
[62]	Neonatal rat ventricular myocytes	Brief SI/SR or PPC	Hypoxia (98.5% N_2–1% CO_2, 0.5% O_2)	Calcein/ethidium bromide+ LDH measurements	Enzyme release, cell viability, HSP70 expression, PKC translocation
[63]	Human ventricular myocytes	Brief SI/SR	Hypoxia (95% N_2–5% CO_2)+ glucose-free medium	Trypan blue staining	Cell viability
[64]	Chick embryo ventricular myocytes	Brief SI/SR	Hypoxia (100% N_2)+ glucose-free medium	Trypan blue staining+ creatine kinase measurements	Enzyme release and cell viability
[65]	Chick embryo ventricular myocytes	Brief hypoxia (95% N_2–5% CO_2) without glucose+SR	Hypoxia (80% N_2–20% CO_2)+glucose-free medium+hyperbaric acidosis, and high K^+	Propidium iodide+ DCF fluorescence	Cell viability and ROS generation
[66]	Neonatal mouse myocytes	Brief anoxia (0% O_2)+ SR, or PPC	Hypoxia (99% N_2–1% CO_2)+glucose-free medium	Calcein and ethidium homodymer	Cell viability
[38]	Adult rat myocytes	PPC	Hypoxia (95% N_2–5% CO_2)+glucose-free medium	Propidium iodide, LDH release, and morphology	Enzyme release, cell viability, and ultrastructure
[67,68]	Neonatal rat ventricular myocytes	Brief hypoxia (94% N_2–1% O_2–5% CO_2)+SR	Hypoxia (94% N_2–1% O_2–5% CO_2)+ glucose-free medium	MTS staining and LDH measurements	Enzyme release and cell viability, mRNA levels (HSP70, Glut4, Glut1)
[69]	Chick embryo ventricular myocytes	Brief SI/SR, or PPC	Hypoxia (95% N_2–5% CO_2)+glucose-free medium	Syntox green staining and DAF-2 fluorescence	Cell viability and NOS production
[22]	Rabbit ventricular myocytes	Brief SI (pH 7.4, normal K^+, no lactate)+SR	Hypoxia (100% N_2)+ glucose-free medium+ pH 6.5+high K^+ and lactate	Trypan blue staining	Cell viability
[70]	Adult rat ventricular myocytes	Brief SI/SR	Hypoxia (100% N_2)	Trypan blue staining and LDH measurement	Enzyme release, cell viability, apoptosis
[71]	Adult rat myocytes	Brief SI/SR (2 cycles), or PPC	Hypoxia+Na-dithionite+ glucose-free medium	Cell injury not assessed	Rigor contracture
[72]	Neonatal rat myocytes	3-cycle SI/SR or PPC	Hypoxia (100% N_2)	Propidium iodide	Cell viability

DCF = 2′,7′-dichlorofluorescein; DAF-2 = Diaminofluorescein-2 diacetate; LDH = lactate dehydrogenase; SR = reoxygenation.

without lactate, and balanced to a physiological pH (7.4) to simulate the mild (non damaging) ischemic episode, followed by a 10 min reperfusion (reoxygenation) period in culture medium 199. We have surrounded the cardiomyocytes during the simulated long (index) ischemia with a solution with low pH (6.5), high K^+ concentration (12 mmol/L) and lactate concentration (20 mmol/L) as occurs in the interstitium of myocardium in vivo during no-flow ischemia. The lack of glucose and fatty acids (which are not supplied in the media) together with the severe hypoxia forces the cardiomyocytes to rely largely on glycogenolysis for ATP production and the acidotic environment and high lactate concentrations tend to inhibit anaerobic glycolysis. We have found that with this approach we have achieved about 60% cardiomyocyte cell death, as assessed by *iso*-osmotic trypan blue staining, for a 60 min index-ischemia followed by a 60 min simulated reperfusion, achieved by replacing the "ischemic" culture medium with normoxic culture medium with physiological pH, lactate, and K^+ concentrations, the same cardiomyocyte mortality as with 45 min of simulated ischemia in the pelleted fresh cardiomyocyte model under similar conditions of simulated reperfusion [22]. It is noteworthy that none of the cultured cardiomyocyte models we cite use irreversible metabolic inhibitors such as cyanide. Our view is that the use of any irreversible form of metabolic inhibition is inappropriate in simulating ischemia because it is unnecessary and complicates interpretation of cell mortality on simulated reperfusion.

Trypan blue exclusion has been used extensively in cultured cardiomyocyte models to assess cell viability but propidium iodide [65] and ethidium bromide [62] have also been used. These latter stains penetrate the cell through the damaged cell membrane and stain the nucleus. Enzyme release (lactate dehydrogenase or creatine kinase) has also been used by several investigators as an alternative measurement of cardiomyocyte injury (Table 2). Enzyme release measurements have the advantage of avoiding the assessment of large number of cells by dyes although, in our view, the direct verification of cell mortality by trypan blue or other staining techniques remains appealing. The use of dyes which enter the cardiomyocyte through damaged, permeable cell membrane, or enzyme release from the cell to mark cell mortality, relates to one mechanism of cell death, oncosis (derived from the Greek word "onkos" for swelling) in which there is cytoplasmic swelling, with intracellular organelles swelling, increased cell membrane permeability and blebbing and ultimate cell membrane rupture [73]. In apoptosis, the cell cytosol shrinks, there is marked chromatin condensation and the cell breaks up into apoptotic bodies [73]. Used correctly, "necrosis" refers to changes secondary to cell death by any mechanism but is often used to refer implicitly to oncotic cell death in contradistinction to apoptosis. In acute myocardial ischemia of sufficient duration to cause substantial injury (occurring in all models to demonstrate the efficacy of IPC), the substantial majority of cell death, about 90%, is by oncosis [74].

Apoptosis can also be studied in isolated cardiomyocytes, but requires several hours after simulated reperfusion become evident, longer than the observation interval in most studies. It is noteworthy that because of the non-specificity of TUNEL staining for apoptosis there has been a tendency to attribute apoptosis to cardiomyocytes dying by oncosis in the whole heart [75]. This is probably also true in isolated cardiomyocytes.

Other endpoints than cell viability can be studied in cultured cardiomyocytes such as rigor contracture [71] and generation of reactive oxygen species [76].

Cell culture allows the possibility of genetic manipulations to precisely target the "molecular machinery" of preconditioning and opens up exciting possibilities to further knowledge in this field, examples of which follow.

7. Using cardiomyocytes to study cell volume regulation in ischemic preconditioning

We have hypothesized that ischemic preconditioning triggers the activation of cell volume regulatory mechanisms that operate throughout the long (index) period of ischemia and reperfusion, thus extending the duration of the index ischemia that causes lethal cell swelling and thus results in myocardial protection. This hypothesis is based on the fact that metabolites (osmolytes) accumulate in cardiomyocytes during ischemia, drawing in osmotically obligated water causing cell swelling. Such swelling is limited during no-flow ischemia because osmolytes also accumulate in the extracellular space tending to equilibrate with those within cardiomyocytes. However, on reperfusion in vivo, cardiomyocytes are exposed to *iso*-osmotic perfusion which rapidly washes out the extracellular osmolytes reimposing an osmotic gradient which causes abrupt, further swelling which may rupture already weakened cell membranes. If the cardiomyocyte retains its ability to correct cell swelling by pumping out osmolytes, of which Na^+, Cl^- and K^+ are important constituents, together with osmotically obligated water, then potentially fatal cell swelling during ischemia and on reperfusion may be mitigated and protection ensues.

We have measured cell volume changes in isolated cardiomyocytes in primary culture under a variety of conditions to test this hypothesis. In a collaborative study with Diaz et al. [20], both freshly isolated pelleted cardiomyocytes and cultured cardiomyocytes were used to establish that (i) IPC triggers a cell volume regulatory mechanism that reduces cardiomyocyte swelling during the subsequent index ischemia; (ii) this reduction in ischemic cell swelling is of sufficient magnitude (an osmolar gradient equivalent of 50–60 mOsM) to account for the protection of IPC; and (iii) the molecular mechanism that mediates IPC also mediates cardiomyocyte volume regulation, specifically

regulatory volume decrease to an osmotic challenge, under normoxic conditions.

No doubt, decreased cardiomyocyte swelling during ischemia and reperfusion is not the only protective mechanism in preconditioning; however, it appears to make a substantial contribution. Logically, the ion channels which are responsible for osmolyte export in cardiomyocyte regulatory volume decrease are candidate end effectors of preconditioning. This has been the basis of our interest in sarcolemmal Cl^- and K^+ channels [18–20,22].

8. Manipulating gene expression in cardiomyocytes to study ischemic preconditioning

Genetic manipulation is a particularly powerful tool to study mechanisms in preconditioning because it bypasses the non-specificity and undesirable side effects of pharmacological manipulations. The most obvious strategy is to study isolated cardiomyocytes from "knockout" mice in which the production of functional copies of a particular protein have been substantially reduced or eliminated. Study in freshly isolated cardiomyocytes suffices for this purpose with simple cell viability as the endpoint. A good example is the study of Smith et al. [53] which demonstrated that ischemic and pharmacological preconditioning protected wild-type cardiomyocytes but not those of STAT-3 transcription factor deficient mice. Li et al. [55] used freshly isolated cardiomyocytes from heterozygous connexin 43 deficient mice (the homozygote being lethal) to show that, in contrast to wild-type cardiomyocytes, preconditioning did not protect the connexin 43 depleted cardiomyocytes [77], the same result they had obtained in whole mouse hearts [23]. Their use of isolated cardiomyocytes was particularly instructive in that it excluded a role for intercellular communication through gap junctions of which, in ventricular cardiomyocytes, connexin 43 is an essential component.

Another approach to controlling gene expression is to transfect cardiomyocytes in cell culture with a recombinant virus which will express one or more mutants of the gene of interest. Ping et al. [78] and Li et al. [79] used this approach to study the role of the PKC epsilon isoform as a mediator of preconditioning. Recombinant adenoviruses encoding either the rabbit wild-type PKCε gene or the dominant negative mutant PKCε gene were produced by cloning the PKCε cDNA into the human adenoviral type 5 genomic DNA. It was sufficient to transfect the cultured cardiomyocytes for 18 h (overnight) to achieve adequate PKCε transgene protein expression as determined by immunoblotting and as borne out by the results of their experiments on cardiomyocytes protection after a total of 48 h in culture. In these studies, ischemia was simulated by means of severe hypoxia in an atmosphere of 85% nitrogen, 10% hydrogen and 5% CO_2 in a glucose-free buffer solution at pH 6.5 containing 20 mM lactate [78]. Reperfusion was simulated by reoxygenation (95% air–5% CO_2) with the addition of normal culture medium for 1 h. Cellular injury was evaluated through LDH release expressed as a percentage of total LDH activity released when, at the end of the experiment, all cells were lysed and LDH measured.

We have used recombinant adenovirus transfection of cultured cardiomyocytes to evaluate the role of the voltage-independent K^+ channel I_{K1} in IPC. Our interest in I_{K1} flowed from our interest in cardiomyocyte swelling and rupture in ischemia/reperfusion injury and the logic that for every negatively charged ion which must exit the cardiomyocyte to remove osmotically obligated water and reduce swelling there must be a balancing positively charged ion moving in the same direction to maintain cell membrane electroneutrality. The I_{K1} channel is predominantly composed of Kir2.1 and Kir2.2 heterotetramers [80] so that two Kir2.1 subunits and two Kir2.2 subunits together form the pore. Substitution of just one amino acid of either Kir2.1 (C122S) or Kir2.2 (C123S) in the pore forming region was sufficient to knock down the I_{K1} current by about 50% in cultured cardiomyocytes. The combination of Kir2.1 and Kir2.2 dominant negatives (DNs) knocked down the current by 70% or more. To study the effect of the dominant negative Kir2.1 and Kir2.2 genes on ischemic preconditioning, it was important for us to overexpress a reporter gene, for which we chose enhanced green fluorescent protein (EGFP). This allowed us to adjust the viral transfection load to achieve over 98% of transfection efficiency (as determined by the green fluorescence observed in cells) but without a loss of cell viability. Ischemia was simulated as we have described earlier in this review, in a similar fashion to that used by Ping et al. [78], as was reperfusion, but cell injury was evaluated by trypan blue exclusion not LDH release. After establishing that IPC protection was not affected by the EGFP reporter gene, we found that adenovirus (Ad) overexpression of either of the dominant negatives (AdEGFP Kir2.1DN or AdEGFP Kir2.2DN) was sufficient to abolish the IPC protection against cell death in cultured cardiomyoctes [22].

In manipulating gene expression in isolated cardiomyocytes it is, of course, important to assess the consequences to the normal function of the target protein. This we did for I_{K1} using patch-clamp measurements of current density [80,81]. Protein expression may change in cultured cardiomyocytes and amplitude of I_{K1} current is diminished in quiescent cardiomyocytes after only 2 to 3 days when compared to I_{K1} current in freshly isolated cardiomyocytes [82]. We measured this reduction to be about 50% but were able to document that IPC protection was as potent as we observed in freshly isolated cardiomyocytes. Thus, we found it useful to compare results in fresh versus cultured cardiomyocytes. We used Ba^{2+}, a relatively non-specific blocker of K^+ currents in the fresh cardiomyocytes, with simulated ischemia by pelleting and obtained supportive results. However, it was only through molecular targeting of I_{K1}, through the knowledge of the structure of this ion channel

that allowed the production of the dominant negative genes for its subunits, which allowed us to achieve complete specificity for I_{K1}.

Another example of adenovirus-mediated gene expression applied to cultured cardiomyocytes is the study of Date et al. [67] in which expression of the anti-apoptotic protein p35, a potent inhibitor of all mammalian caspases except caspases 5 and 9, was demonstrated to reduce cellular levels of reactive oxygen species (ROS), and reduce caspase activity and apoptosis. This study did not directly address preconditioning and therefore, is actually outside the parameters we set for inclusion in this review. However, from a methodological perspective it illustrates how gene expression manipulation can be combined with visualization of intracellular ROS through dichlorohydrofluorescein diacetate (DCFDA) fluorescence, enzyme activity (caspases, antioxidant enzymes) measurements and visualization of apoptosis through TUNEL staining. The Vanden Hoek et al. [65] study in cultured chick embryonic ventricular cardiomyocytes is another example of fluorescence visualization of ROS generation, important in preconditioning signaling [69,76] and they directly addressed preconditioning.

A further example of adenovirus transfection of cultured cardiomyocytes in preconditioning is from the Uchiyama et al. [70] study in which overexpression of constitutively active Akt, versus dominant negative Akt, was used to examine protection of hypoxic preconditioning against hypoxic/reoxygenation injury of adult rat cardiomyocytes. Cell viability was assessed by trypan blue exclusion and induction of apoptosis, after 120 min of reoxygenation, was evaluated by measurement of caspases 3 and 9, cytochrome *c* release and TUNEL staining.

9. Probing the role of mitochondria in ischemic preconditioning through observations in intact cardiomyocytes

Mitochondria play an important role in ischemic preconditioning [24,83–85]. While insight has been gained from studies of isolated mitochondria [86], there are considerable advantages in studying mitochondria in intact cardiomyocytes under conditions that likely mimic ischemia and reperfusion more faithfully than the somewhat arbitrary manipulations of the chemical milieu in which preparations of pure mitochondria are studied.

Liu et al. [48] made use of the autofluorescence of NADH and flavoproteins in the mitochondrial matrix to sense the mitochondrial redox state in cultured cardiomyocytes and found that diazoxide induced a reversible increase in flavoprotein oxidation and cardiomyocyte protection using the pelleting model of Vander Heide et al. [32], and 5-hydroxydecanoic acid inhibited both the flavoprotein oxidation changes and cardiomyocyte protection against ischemic cell death. The mitochondrial fluorescence measurements were interpreted to indicate the opening of the putative mitochondrial ATP sensitive K^+ channel (mito-K_{ATP}). This interpretation has recently been questioned by Hanley and Daut [83] but, despite this aspect of the broader controversy regarding mitoK_{ATP}, there appears to be an agreement that flavoprotein fluorescence is a potentially valuable indicator of the rate of electron transfer by the respiratory chain.

Another approach to the mitochondria through fluorescence measurements has been used by Hausenloy et al. [71] to address the role of the mitochondrial permeability transition (mPT) in cultured adult rat cardiomyocytes. These investigators incubated rat ventricular myocytes in the fluorescent dye TMRM which accumulated selectively in mitochondria, then used laser illumination of this dye in a confocal microscope to generate ROS within the mitochondria and provoke the mPT with loss of this dye into the cytosol. They also examined the redistribution of calcein-AM fluorescent dye from the mitochondria into the cytosol as an independent method to verify the mPT. They found that hypoxic preconditioning for two periods of 10 min at 37 °C, with intervening 30-min reoxygenation in restoration buffer, significantly reduced the time required to induce opening of the mitochondrial permeability transition pore (mPTP) as did pharmacological preconditioning with diazoxide and nicorandil and pharmacological inhibitors of mPTP opening such as cyclosporine A. Of note, cell viability as assessed by trypan blue or another dye, such as ethidium bromide or propidium iodide, was not assessed. Rather, the time required to induce rigor contracture was an endpoint.

10. The future for cardiomyocytes in ischemic preconditioning research

Efforts to understand ischemic preconditioning at a mechanistic level are at a stage where the triggers and mediators of preconditioning need to be linked to the end effectors. We believe these discoveries will increasingly require the use of isolated cardiomyocytes and will rely, in large part, on tracing the signaling pathways to their end destinations in the cardiomyocytes using techniques such as immunoblotting and immunoprecipitation (i.e., pulldown assays) of sarcolemma and mitochondria, combined with immunocytochemical visualization, to reveal kinase interactions with multi-protein signaling complexes [87], for example, PKCε and Src tyrosine kinase [88], and key end effector proteins.

Isolated cardiomyocytes, and increasingly those in primary cell culture which offer the opportunity for repeated visualization of molecular events in the same cells, provide an essential platform for these studies and should make a critically important contribution to future preconditioning research and, more generally, the mechanistic study of the pathophysiology of myocardial ischemia and reperfusion.

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