Preconditioning the Myocardium: From Cellular Physiology to Clinical Cardiology

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Yellon, Derek M., and James M. Downey. Preconditioning the Myocardium: From Cellular Physiology to Clinical Cardiology. Physiol Rev 83: 1113–1151, 2003; 10.1152/physrev.00009.2003.—The phenomenon of ischemic preconditioning, in which a period of sublethal ischemia can profoundly protect the cell from infarction during a subsequent ischemic insult, has been responsible for an enormous amount of research over the last 15 years. Ischemic preconditioning is associated with two forms of protection: a classical form lasting ~2 h after the preconditioning ischemia followed a day later by a second window of protection lasting ~3 days. Both types of preconditioning share similarities in that the preconditioning ischemia provokes the release of several autacoids that trigger protection by occupying cell surface receptors. Receptor occupancy activates complex signaling cascades which during the lethal ischemia converge on one or more end-effectors to mediate the protection. The end-effectors so far have eluded identification, although a number have been proposed. A range of different pharmacological agents that activate the signaling cascades at the various levels can mimic ischemic preconditioning leading to the hope that specific therapeutic agents can be designed to exploit the profound protection seen with ischemic preconditioning. This review examines, in detail, the complex mechanisms associated with both forms of preconditioning as well as discusses the possibility to exploit this phenomenon in the clinical setting. As our understanding of the mechanisms associated with preconditioning are unravelled, we believe we can look forward to the development of new therapeutic agents with novel mechanisms of action that can supplement current treatment options for patients threatened with acute myocardial infarction.
I. INTRODUCTION: MYOCARDIAL PRECONDITIONING

Acute coronary occlusion is the leading cause of morbidity and mortality in the Western world and according to the World Health Organization will be the major cause of death in the world as a whole by the year 2020 (238). Although the management of this epidemic will center on the development of effective primary prevention programs, the impact of these strategies may be limited, particularly in the developing countries. There is an urgent need for effective forms of secondary prevention and, in particular, treatments which will limit the extent of an evolving myocardial infarction (MI) during the acute phase of coronary occlusion. The death of myocardium represents a catastrophic event as dead myocytes are not replaced by division of surviving myocytes. Preserving the viability of ischemic myocardium therefore has been recognized as a major therapeutic target.

In the early 1970s, attention was first focused on this problem in the animal laboratory. Maroko et al. (215) proposed that a variety of interventions at the time of coronary occlusion could reduce the size of the resulting infarct in the open-chest dog. At that time the models for evaluating infarct size were very crude, and none of the original interventions proposed, such as beta blockers (215), glucose-insulin-potassium (217), or hyaluronidase (216), ever proved to be effective. Nevertheless, those early studies initiated the search for interventions that could limit infarct size in the clinical setting. Jennings and Reimer (159) demonstrated that reperfusion was essential to protecting the ischemic myocardium, and soon thereafter, the introduction of thrombolytic therapies became routinely available. While reperfusion therapy clearly was reducing infarct size, as demonstrated by the extensive NIH-sponsored Thrombin Inhibition in Myocardial Ischemia (TIMI) trials, it also became clear that reperfusion had not eliminated infarction. Unfortunately, myocardium begins to die in minutes; however, dissolution of the offending thrombus and subsequent reperfusion usually requires hours to accomplish so there was still a need for an infarct sparing intervention. The quest was hampered by the fact that scientists did not (and still do not) know what the lethal event is in the ischemic heart. A number of candidates such as anti-inflammatory agents and free radical scavengers were examined through the early 1980s and were seldom adequately controlled. In retrospect, most of those studies, including some published by the authors of this review, were probably simply false positives.

Perhaps the single greatest advance in our understanding of the cell survival machinery was the discovery in 1986 by Murry et al. (239) of an intrinsic mechanism of profound protection, which they termed ischemic preconditioning (see Fig. 1). In this seminal paper, they showed that four cycles of 5 min of ischemia with intermittent reperfusion were shown to limit infarct size by 75%, an amount of protection heretofore unheard of. More importantly in the subsequent years that followed, the anti-infarct effect of preconditioning could be reproduced by all who tried it (for a review of the early studies, see Ref. 192). For the first time it was shown that infarct size limitation was theoretically possible. Indeed, so powerful was the observed protection that this phenomenon has been recognized as “the strongest form of in vivo protection against myocardial ischemic injury other than early reperfusion” (175). It appeared therefore that all that remained was to investigate the mechanism associated with the profound protection in order for pharmacological mimetics to be developed that could ultimately be used in the clinical setting. At the time of writing this review there have been in excess of 2,000 papers published on this subject.

![Figure 1](http://physrev.physiology.org/)
II. NATURAL HISTORY OF CLASSICAL ISCHEMIC PRECONDITIONING

A. Triggers Versus Mediators

When considering preconditioning it is useful to think in terms of triggers, mediators, memory, and end-effectors. The preconditioning ischemia triggers a change in the physiology of the heart, rendering it very resistant to infarction. We know that a series of signal transduction pathways carry the signal for protection, and those presumably terminate on one or more end-effectors. The end-effectors actually cause the protection during the lethal ischemic insult (index ischemia) and/or the subsequent reperfusion period. Somewhere in the signal transduction pathways between the trigger signal and the end-effector is a memory element that is set by the preconditioning protocol and keeps the heart in a preconditioned state. All steps distal to the memory step including the end-effector can be classified as mediators since they exert their activity only after the index ischemia has begun, and those proximal to the memory element can be considered triggers because they exert their effect only prior to the index ischemia.

B. Duration of Effect

Ischemic preconditioning has been demonstrated in all animal species studied to date including the chicken (202), dog (239), mouse (322), pig (301), rabbit (72), rat (201), and sheep (53). All of our knowledge about preconditioning is empirically derived from studies in a variety of species including humans. Although it is assumed that preconditioning’s mechanism is common to all species, there have been obvious mechanistic discrepancies among the various reports, and some of them could well be related to species differences. Some of these differences are obvious such as the role of xanthine oxidase as discussed below, but the reader should note the species being studied and consider the possibility that any reported mechanism may be species specific and more importantly may not be relevant to human heart. In terms of the extent of the protection observed, it must be noted that preconditioning’s protection is lost when the ischemic insult was extended to 3 h, indicating that reperfusion after the lethal ischemic insult is an absolute requirement (239).

These observations indicate that ischemic preconditioning delays rather than prevents cell death. Most animal studies to date would suggest that preconditioning acts as if it had reduced the duration of the ischemic insult by ~20–30 min. In primate myocardium, which for unknown reasons infarcts much more slowly, that benefit may be much longer (309). Murry and colleagues also found that the protection seen with preconditioning was independent of collateral flow indicating that the ability of the heart to withstand ischemia had been directly modified. The cardioprotection described by Murry et al. (239) has become known as “classic” or “early” ischemic preconditioning.

The preconditioned state is very transient following a preconditioning protocol and lasts for only 1–2 h in anesthetized animals (240, 287, 363) and is lost somewhere between 2 and 4 h in conscious rabbits (52). How the heart remembers that it is preconditioned is another mystery that has resisted laboratory investigation. Thornton et al. (340) reported that protein synthesis inhibition with either actinomycin D or cycloheximide did not block preconditioning’s protection seemingly eliminating gene expression as a possible mechanism of the memory. Matsuyama et al. (219) also obtained the same result with actinomycin D; however, using a much higher concentration of cycloheximide, they were able to block protection from ischemic preconditioning. They suggested that because cycloheximide only blocks translation of message that preconditioning causes the translation of a preexisting mRNA coding for a protective protein. Since the initial study by Thornton et al. directly confirmed protein synthesis inhibition, the most likely explanation is that the latter study may have suffered from a nonspecific effect due to the high cycloheximide concentration. Also, because the preconditioned state can be achieved within 10 min, it is unlikely that any protein could be expressed in such a short time period. The memory information is probably carried as a reversible posttranslational modification of some preexisting protein (such as a phosphorylation or translocation), but the site of that modification is unknown.

Although the initial window of protection is quite transient, a delayed form of the protection reappears within 24 h of the preconditioning stimulus, which has been referred to as the second window of protection (SWOP) (213). The less robust, although more prolonged, SWOP occurs between 12 and 72 h after a preconditioning stimulus (26) (see Fig. 2). Both classical and SWOP preconditioning share some similarities. In both cases the preconditioning ischemia provokes the release of a number of trigger substances that interact with cell surface receptors, thereby initiating a signaling cascade of events. These triggers appear to be the same in both forms of preconditioning. The time course within which the SWOP confers protection allows for the possibility of new protein synthesis, posttranslational protein modification, and a change in the compartmentalisation of existing proteins. The mechanisms of SWOP will be discussed in greater detail in section ivB.

C. End Points of Preconditioning Studies

1. Infarct size

The original end point used for the assessment of protection from classical preconditioning was infarct size.
(239) expressed as a percentage of the risk zone. Many animals have coronary collateral vessels that provide a low level of irrigation during ischemia. Collateral flow opposes infarction causing an inverse relationship between collateral flow and infarct size. In animals with preformed collaterals such as the dog, collateral flow must be taken into account (284). Infarct size is commonly assessed by staining viable tissue with tetrazolium salts. The tetrazolium salts react with NADH and dehydrogenase enzymes staining the tissue (174). Dead cells lose enzyme and cofactor due to membrane failure and thus do not stain. To sufficiently wash out these components 2–3 h of reperfusion are required. The gold standard for infarct size, however, is histological examination of tissue slices after 3 or more days of reperfusion as was used by Murry et al. in their original description of preconditioning (239). Under these conditions, classical preconditioning has limited infarct size in every species tested.

2. Stunning

Whether preconditioning attenuates stunning has not been totally resolved. Stunning refers to a loss of contractility that immediately follows a sublethal ischemic insult. Unlike the infarcted heart, the stunned myocardium recovers fully in a day or two. For a review on the stunned myocardium, see Reference 48. In most species an ischemic insult of 15 min or less stuns the heart but does not cause infarction. Ovize et al. (258) studied the dog heart and concluded that prior preconditioning did not influence the degree of recovery. This conclusion was based on an analysis in which postischemic function was related to the level of collateral blood flow in each heart. The improved recovery in preconditioned hearts was attributed to better perfusion during ischemia rather than preconditioning. Examination of those data reveals, however, that there was no significant correlation between collateral flow and recovery of function in two of the three experimental groups, indicating that the assumption regarding the influence of collateral flow could not be supported. Hence, the authors’ conclusion may not be firm, and preconditioning rather than higher collateral flow may well have protected against stunning in that study. Jenkins et al. (158) noted that preconditioning did not affect stunning in rabbits, a species whose hearts are deficient in xanthine oxidase. Landymore et al. (190) noted that preconditioning prevented stunning after cardiac transplantation in sheep, but it is not known whether sheep hearts contain xanthine oxidase. Finally, Sekili et al. (307) noted that when dog hearts were pharmacologically preconditioned with a transient infusion of adenosine that no protection against stunning could be observed. The pharmacological preconditioning would not have attenuated adenosine release on a subsequent coronary occlusion (121). If classical preconditioning has an antistunning effect it must be small. That is surprising because SWOP reportedly has a robust antistunning effect in both rabbits (47) and pigs (334).

3. Recovery of mechanical function

Postischemic recovery of contractile function is a commonly used end point for ischemic preconditioning in the isolated rat heart (22, 61, 380). However, recovery of mechanical function after an ischemic insult is influenced by both a combination of the number of surviving myocytes and the degree to which they have been stunned. It is well appreciated that the effect of preconditioning on recovery of function is much less pronounced in species other than the rat (318).

Gelpi et al. (117) proposed that the antistunning effect in the rat might be the result of altered adenosine metabolism in the rat’s heart. Free radicals have been shown to strongly contribute to stunning of reperfused myocardium (48), and rat hearts are rich in xanthine oxidase (97). Adenosine released during ischemia is converted to inosine and then hypoxanthine. Upon reperfusion, hypoxanthine is oxidized by xanthine oxidase producing injurious free radicals that stun the heart (51, 62, 64). Preconditioning the heart with a brief period of ischemia followed by reperfusion will greatly attenuate the amount of adenosine released during the next ischemic episode (364), which in turn would reduce the amount of xanthine oxidase-mediated free radical production and...
Thus stunning. Indeed, Gelpi et al. (117) found that the xanthine oxidase inhibitor allopurinol improved postischemic function and greatly attenuated the improvement from preconditioning in the rat model.

In rabbit hearts, which do not contain xanthine oxidase, preconditioning had no effect on postischemic function. To further complicate the situation, the mechanism by which preconditioning attenuates purine release seems to be unrelated to that used by the anti-infarct effect (121) so that extrapolation of data from the rat recovery of function model to preconditioning in humans may be very misleading.

The overall aim of myocardial salvage is to improve ventricular function. Oddly enough few studies have tested whether preconditioning really does yield a stronger heart in the animal laboratory. Cohen et al. (76) measured ventricular wall motion in conscious rabbits subjected to regional ischemia/reperfusion. Not only did preconditioning reduce infarct size, but it also improved wall motion in the ischemic zone. It took at least a day for stunning to subside however before the benefit could be appreciated.

4. Arrhythmias

Ischemic preconditioning has also been reported to alter the incidence of ischemia and reperfusion-induced arrhythmias in dogs (367) and rats (310). The experience with most investigators studying other species has been that preconditioning either has little effect on arrhythmias or actually exacerbates it (256). Free radicals are also known to lead to the genesis of arrhythmias in the heart (187), and it is likely that antiarrhythmic effect of preconditioning in dog and rat may again be related to attenuated purine release, although that hypothesis has not been directly tested.

5. Electrocardiographic changes

It must be emphasized that infarct size testing is the only established end point for preconditioning at this time, and extrapolated findings from recovery of contractile function or arrhythmias need to be interpreted with caution. Unfortunately, studies in humans have forced investigators to examine surrogate end points such as electrocardiographic changes. Cribier et al. (80) noted that in patients undergoing coronary angioplasty that the S-T segment voltage was much lower during the second coronary occlusion than on the first. He concluded that this reflected the protection of preconditioning from the first balloon inflation. A number of drugs were subsequently tested in this setting to see if they could mimic or block preconditioning in humans (for a review, see Ref. 344). One criticism of the technique was that the reduced S-T segment voltage might only have reflected opening of collateral vessels between the inflations. Shattock et al. (308) disproved that hypothesis by showing that the same response could be seen in pig heart, which is collateral deficient. It was later shown that the S-T segment changes were influenced by surface ATP-sensitive potassium (KATP) channels while protection from preconditioning was influenced by those in the mitochondria (41). It would appear that preconditioning opens both populations of channels while only the mitochondrial population acts to protect (see below). Thus S-T segment changes may not be a reliable end point for preconditioning studies either.

D. The Preconditioning Stimulus

Ischemic preconditioning requires a brief period of ischemia followed by reperfusion to trigger the response. The minimum period of reperfusion required giving protection after the preconditioning ischemia lies between 30 s and 1 min (5). Preconditioning was originally reported to be an “all or none” phenomenon. Li et al. (200) compared 1, 6, and 12 cycles of 5-min coronary occlusions in the dog and found no differences in the protection. Another report (363) found no differences between one and two 5-min cycles of preconditioning in the rabbit. A similar observation was made in ex vivo human cardiac tissue (236). On the other hand, studies using in vivo rat (24a) or pig (304) models found that the resulting infarct size varied with the strength of the preconditioning stimulus, suggesting a graded response. Off-on systems are rare in nature, so more than likely preconditioning is merely following a very steep dose-response curve. Once a maximal response is achieved, further stimulation has no additional effect, giving the impression of an all or none system. Many studies take advantage of this very steep dose-response relationship. A single 2-min coronary occlusion will not precondition the rabbit as it is below threshold (363). However, in the presence of an intervention that potentiates the triggers of preconditioning, such as angiotensin-converting enzyme (ACE) inhibitors which augment interstitial bradykinin levels (155, 227) or agents that augment adenosine such as acadasine (52), preconditioning will occur.

E. Preconditioning in Other Organs

Preconditioning was first described in the heart but since then it has been seen in various forms in a variety of organs. Classical preconditioning is seen in skeletal muscle (262), and its mechanism seems to be virtually identical to that seen in the heart directly protecting the parenchymal cells (261). Classical preconditioning has also been described in the gut (152) and in the kidney.
In the intestine, the microcirculation is the primary target for ischemic injury while in the kidney it is the proximal tubular cells. Yet the result is the same in all three tissues, a rapid protection against cell death during ischemia. In the brain (171), preconditioning is only protective in a second window-type setting a day after the preconditioning stimulus. Thus it would appear that preconditioning represents a generalized adaptation to protect a wide variety of cells against stressful stimuli such as ischemia.

F. Remote Preconditioning

One form of preconditioning that is poorly understood is remote preconditioning. In 1993 Przyklenk et al. (278) reported that preconditioning one region of the dog heart caused protection in a remote region. They hypothesized that a circulating humor or perhaps a neural reflex triggered protection in the remote region. Similarly mesenteric artery occlusion was also seen to result in protection of the rat heart, further supporting the hypothesis (368), and it was subsequently found that blockade of opioid receptors in the rat blocked that response (266), suggesting either a circulating endorphin or neural link. Nakano et al. (246) tried to duplicate the effect in a rabbit model where a region of the heart was preconditioned in situ and then the heart was removed and exposed to global ischemia. The amount of infarction was measured in both the preconditioned and the nonpreconditioned regions, but no differences were seen. It was concluded that preconditioning one region of the heart does not necessarily precondition the remote regions in all species.

III. CELLULAR MECHANISMS OF CLASSICAL PRECONDITIONING

A. Trigger Mechanisms

In 1991 Downey and colleagues (206) found that the adenosine A1 receptor acts to trigger ischemic preconditioning’s protection in the rabbit heart, revealing that ischemic preconditioning is receptor mediated. Blockers of the A1 receptor eliminated preconditioning’s protection while transient exposure to an A1 agonist would confer the protection. Shortly thereafter, Banerjee et al. (22) reported a similar situation with norepinephrine through the α-receptors in the rat heart. We now know that any G_i-coupled receptor can trigger the preconditioned state, and in fact, multiple receptors work in parallel to provide redundancy to the preconditioning stimulus. During a brief ischemic period, the heart appears to release adenosine, bradykinin, norepinephrine, and opioids. Population of their respective receptors then triggers the preconditioned state through activation of G_i protein. As a result, blockade of the bradykinin receptor in the rabbit blocks protection from a single cycle of preconditioning but not from multiple cycles (122). Thus blockade of a single receptor type acts only to raise the ischemic threshold required to trigger protection rather than completely block it (see Fig. 3). The cardiac myocytes express other G_i receptors such as the angiotensin AT_1, the endothelin ET_1, and the muscarinic receptors that can also trigger a preconditioned state but do not seem to participate in ischemic preconditioning simply because agonists to those receptors are not produced in the ischemic myocar-

FIG 3. An example of how multiple receptors act in parallel in ischemic preconditioning. In the first panel, 5 min of ischemia reaches the threshold for protection, but in the second panel, 3 min of ischemia does not. In the third panel, blocking the bradykinin B2 receptor with HOE 140 causes a 5-min ischemic period to become nonprotective because it can no longer reach the threshold. Conversely, augmenting bradykinin’s contribution with an angiotensin converting enzyme (ACE) inhibitor, which prevents bradykinin breakdown, allows 3 min of ischemia to reach a protective threshold. [Modified from Goto et al. (122).]
dium. For a detailed review of this receptor interplay, see Reference 71.

Free radicals also act as trigger mechanisms to preconditioning. Treatment with a free radical scavenger can raise the threshold of preconditioning, and a free radical generator can trigger a preconditioned state (17, 354). It is thought that the free radicals act to directly activate protective kinases (120, 369). In species whose hearts are rich in xanthine oxidase, the free radicals may derive directly from xanthine oxidase’s action on purine catabolism. This conceivably could lead to a nonreceptor-mediated triggering of protection. In other species, such as the rabbit heart, the free radicals seem to have a much more complex origin, as will be discussed later in this review.

Several other non-receptor-triggered forms of preconditioning have been described. Ashraf’s group (230) has found that a short period of elevated Ca2+ in the coronary perfusate will put the isolated rat heart into a protected state that appears to be the same as that from ischemic preconditioning as it is protein kinase C (PKC) dependent (see sect. mB1). Furthermore, they found that the calcium channel blocker verapamil could block not only Ca2+-induced preconditioning in the rat heart but ischemic preconditioning as well (229). A similar observation was made in an in situ dog model where intracoronary calcium chloride triggered protection and ischemic preconditioning could be blocked with a sodium/calcium exchanger blocker (279).

A transient period of hyperthermia has also seemed to be cardioprotective with both a transient early phase and a prolonged late phase (395). Whether the early phase uses the preconditioning mechanism is unknown. Stretch of the myocardial fibers is reported to precondition the dog heart (257), and the protection could be blocked by gadolinium, a blocker of stretch-activated channels. Transient exposure to ethanol can trigger the preconditioned state, but interestingly, if it continues to be present during the index ischemia, it will block its own protection (185). Other forms of preconditioning such as pacing (183, 263) and hypoxia (372) likely induce their protection through release of receptor ligands due to the negative energy balance.

Although nitric oxide (NO) has been linked to the trigger and end-effector phases of delayed preconditioning (see later), evidence for its role in early preconditioning is limited if not controversial (210, 247, 277, 379, 384). It appears that in early preconditioning, NO may lower the threshold for the protection observed, even though in itself it may not be a direct trigger of early preconditioning (36). Importantly, because exogenous (not endogenous) NO has been shown to trigger preconditioning both in rabbits (247) as well as in the endothelial NO synthase (eNOS) knock-out mouse (36), it appears as if the downstream targets for NO remain intact and as such may be pivotal in mediating the protection.

B. Mediators: Signal Transduction Pathways

1. PKC

The role of PKC in preconditioning was codiscovered by Mitchell et al. (228) and Ytrehus et al. (408) in 1994. It can be shown that blockade of PKC eliminates the protection from a preconditioned heart but has no effect on a nonpreconditioned heart. Similarly, activation of PKC by phorbol esters will put the heart into a preconditioned state. PKC is a serine/threonine kinase that is activated by lipid cofactors derived from breakdown of membrane lipids by phospholipase C. There are multiple isoforms of PKC in the heart, each having a similar substrate specificity. They can be broken down into the classical isoforms α, β, and γ, which are dependent on both the lipid cofactor diacylglycerol (DAG) and calcium. The novel isoforms, δ, η, and ε, are calcium independent needing only DAG. Finally, the atypical isoform ζ requires neither DAG nor calcium. The PKC isoforms are thought to achieve specificity by their peculiar physical translocation to docking sites. Mochly-Rosen and colleagues (161) discovered that each isoform will dock on a unique binding protein called a receptor for activated C kinase (RACK) when activated. These RACKs are strategically located only on certain organelles within the cell to bring the PKC isoform in proximity to a specific substrate protein. Binding to the RACK completes the activation and causes the isoform to phosphorylate any nearby substrate. It is thought that only certain isoforms participate in preconditioning. The ε (125, 203, 274), the δ (412), and the α (374) isoforms of PKC have all been proposed to be responsible for preconditioning’s protection. The intracellular targets of PKC have not been established.

One of the great mysteries of preconditioning is its memory. Transient activation of the G1-coupled receptors puts the heart into a preconditioned state lasting ~1 h. Receptor occupation is a trigger of the preconditioned state, and thus the critical time for receptor occupation is during the preconditioning ischemia before the index ischemia (204). This is in contrast to administration of a PKC blocker. If staurosporine, a potent PKC blocker, is given with the same schedule, then protection persists. It would be logical that PKC would phosphorylate substrate during preconditioning and then as long as the substrate remained phosphorylated the heart would be protected. Waning of protection would result from dephosphorylation by phosphatases. Unfortunately, the facts do not fit the theory. The kinase activity of PKC can be completely blocked with staurosporine during preconditioning, and the heart still enters a preconditioned state (398). Only if the staurosporine is present during the 30-min index isch-
emia is protection aborted. Thus PKC is a mediator rather than a trigger of protection, and the memory step must reside upstream of PKC activity. One theory of preconditioning’s memory that has never been proven nor disproved is the translocation of PKC hypothesis. Activation of PKCs requires the physical translocation of the enzymes to their docking sites. It was proposed early on that such translocations would position the kinase for early phosphorylation of substrate with a subsequent occlusion (209).

Kitakaze et al. (173) proposed that the memory in preconditioning is due to the activation of 5′-nucleotidase by PKC. When activated the heart would generate more adenosine from the breakdown of ATP during ischemia, and the adenosine would directly protect the heart. In that case the PKC would be the trigger and adenosine the mediator. The evidence supporting this was that blockade of 5′-nucleotidase blocked the protection of preconditioning in a dog model (173). There are several arguments to this hypothesis however. Although a PKC inhibitor could block protection triggered by adenosine in rabbits, an adenosine receptor blocker could not block protection from a PKC activator (149). That would put adenosine upstream of PKC rather than downstream. Subsequently, Silva et al. (315) found that augmenting interstitial adenosine manyfold during ischemia with an adenosine deaminase inhibitor offered no limitation of infarct size in the dog heart, indicating that elevated adenosine during ischemia alone does not protect. Protection must be triggered by adenosine receptor occupation before ischemia. More than likely the blockade of 5′-nucleotidase in Kitakaze’s experiment simply removed the adenosine trigger for preconditioning.

2. Tyrosine kinase and the mitogen-activated protein kinases

Other kinases have been identified. Using genistein, a broad spectrum tyrosine kinase inhibitor, Maulik et al. (221) found that it could block protection from ischemic preconditioning and proposed that at least one tyrosine kinase is in the overall pathway. Baines et al. (20) provided evidence that a tyrosine kinase was downstream of PKC; however, several other studies suggest that it (or another one) may be in parallel with PKC in both pig (358) and rat (111). The dynamics of the parallel arrangement are interesting. When a mild preconditioning stimulus such as a single 5-min coronary occlusion is given, either a PKC or a tyrosine kinase blocker on its own will block protection, suggesting that both pathways must be activated to achieve threshold for protection. When a more robust stimulus is used, however, then blocking either pathway alone will not block protection, and both inhibitors must be present. This suggests that either pathway can be protective on its own if stimulated enough (336).

Maulik et al. (221) proposed that the tyrosine kinase in question was the 38-kDa stress-activated mitogen-activated protein kinase (MAPK) (p38 MAPK). What followed has to be one of the most confusing chapters in the ischemic preconditioning stories. Each subfamily of the MAPK family, the 42/44-kDa extracellular receptor kinase (ERK), the 46/54-kDa c-jun kinase (JNK), and the 38-kDa p38 MAPK, has been suggested to play a role in the cardioprotection achieved by ischemic preconditioning (for review, see Refs. 226, 270). All of the MAPKs are activated by dual phosphorylation of a serine and a threonine by a MAPK kinase. The MAPK kinase is a tyrosine kinase and at least the ones targeting p38 MAPK can be blocked by genistein (244).

In isolated rabbit hearts, ERK1 activity reportedly increases only in ischemically preconditioned myocardium (170), but no difference in ERK1 and ERK2 phosphorylation between nonpreconditioned and preconditioned myocardium is detectable in pigs in vivo (33). Like p38 MAPK (see below), ERK can activate MAPKAP kinase 2 α and β, leading to phosphorylation of the small heat shock protein, hsp27 (300).

A causal role of ERK activation in the cardioprotection achieved by ischemic preconditioning is controversial. While PD 98059, an inhibitor of ERK, fails to block the infarct size reduction seen after ischemic preconditioning in isolated rabbit and rat (233) hearts (170), its intramyocardial infusion appeared to abolish ischemic preconditioning’s protection in pigs in vivo (321). It is of interest that ERK1 forms a signaling complex with PKC-ε in the heart along with other MAPK kinases (21). Thus translocations of MAPKs may be involved in the signaling in addition to their phosphorylation.

Both JNK46 and JNK54 are present in the heart (67) and are strongly activated during reperfusion after ischemia. Their activation/phosphorylation during ischemia has been suggested by some studies (33), but in another, a reduction in JNK phosphorylation during ischemia was reported (244). JNK46 activation during no-flow ischemia is most likely mediated by PKC, since activation of JNK46 is completely blocked by chelerythrine, a PKC inhibitor (273). Anisomycin, an activator of both JNK and p38 MAPK, reduces infarct size in rabbits (18) and pigs (23). In isolated rat hearts, blockade of JNK46 with curcumin blocks the infarct size reduction of ischemic preconditioning to a similar extent as blockade of p38 MAPK using SB203580 (294).

Most attention has focused on the p38 MAPK cascade. At least five isoforms of p38 MAPK have been identified, although only p38 α- and β-MAPK are expressed to any degree within the heart (298). Different isoforms of p38 MAPK appear to mediate different biological functions (for review, see Ref. 226). In neonatal rat cardiomyocytes, p38 α-MAPK mediates apoptosis, whereas p38 β-MAPK is antiapoptotic (375).
The activation patterns of p38 MAPK in ischemic heart vary widely between reports. p38 MAPK, like all of the MAPKs, has two amino acids that must be phosphorylated for activation: a threonine residue at amino acid 180 and a tyrosine residue at site 182. This kinase is activated by MAPK kinase (MEK) 3 and 6, which in turn are activated by MAPK kinase kinases (MKK). The phosphorylation of p38 MAPK can be measured with phosphospecific antibodies. p38 MAPK is phosphorylated within minutes during global or regional no-flow ischemia in isolated rat hearts (43, 105, 221), as well as in rat (311, 407), dog (291), and pig hearts in vivo (24, 33).

With prolongation of ischemia, however, the phosphorylation of p38 MAPK may be reduced toward preischemic values, whereas phosphorylation is once again increased upon reperfusion (311, 407). The transient p38 MAPK activation during prolonged ischemia might be related to decreased phosphorylation or increased dephosphorylation by phosphatases (for review, see Ref. 299). In contrast to the above studies, no activation of p38 MAPK by ischemia per se is seen in nonpreconditioned isolated rabbit hearts (221, 244, 377). Following ischemic preconditioning, phosphorylation of p38 during the index ischemia is reported to be increased in isolated rat and rabbit hearts (220, 221, 244, 377), unaltered in pig hearts in vivo (33), and even decreased in dog hearts in vivo (291). Ischemic preconditioning reportedly prevents the ischemia-induced activation of p38 α-MAPK in rat cardiomyocytes (298). At present, it is difficult to see any consistent pattern in the data. Much of the variability in the above reports may stem from technical problems in the processing of the tissue as phosphatases remove these phosphate groups quickly and dephosphorylation can occur with freezing and thawing in the presence of even the best phosphatase inhibitors.

The importance of p38 activation for cardioprotection also is controversial. Rat cardiomyocytes transfected with a dominant negative p38 isoform, which prevents ischemia-induced p38 activation, are more resistant to lethal simulating ischemia (298). Similarly, in isolated rat hearts (300) and pig hearts in vivo (24), blockade of p38 with SB 203580 did not affect the infarct size reduction achieved by ischemic preconditioning, but reduced infarct size in nonpreconditioned hearts. In total contrast, SB 203580 effectively blocked preconditioning’s protection in other cell models (13, 242, 377) and abolished the infarct size reduction of ischemic preconditioning in isolated rat heart (221, 232) and the isolated rabbit heart (245) and dog hearts in vivo (291).

Explanations for the controversial findings might relate to the relative balance between different isoforms of p38 in different species and experimental models as well as the selectivity of different inhibitors in a given dose range. SB 203580, for example, not only inhibits p38 MAPK (81), but also dose-dependently inhibits JNK (68), tyrosine kinases such as p56lck and c-src (370), and cyclooxygenase (50), and it activates c-raf (105). To further complicate matters, some of the above kinases have been implicated in ischemic preconditioning by various investigators.

3. Phosphatidylinositol 3-kinase

Recent evidence has implicated phosphatidylinositol 3-OH kinase (PI 3-kinase) in the signaling of classical preconditioning. Tong et al. (352) were first to report that the PI 3-kinase inhibitor wortmannin could block protection from preconditioning using contractile dysfunction as the end point. That was subsequently confirmed by Mocanu et al. (233) using an infarct size model. PI 3-kinase has been implicated as protective in other cell systems (90). The question with respect to preconditioning is, where is PI 3-kinase located in the signaling system? This is discussed in more detail in section mC5.

C. KATP Channels

1. What are KATP channels?

KATP channels have been shown over the last 10 years to be an important mediator of cardioprotection, and their role in ischemic preconditioning has been demonstrated in whole animals, isolated hearts, and cardiac myocytes. KATP channels were first described by Noma (249) in cardiac ventricular myocytes. These potassium channels are termed ATP sensitive because they are normally inhibited by physiological levels of ATP. KATP channels are modulated by pH, fatty acids, NO, SH-redox state, various nucleotides, G proteins, and various ligands (adenosine, acetylcholine, etc.) (102, 172).

With regard to preconditioning, there is general consensus that KATP channel plays a key role in preconditioning. Gross et al. (129) were the first to propose that opening of the KATP channel was involved in preconditioning’s protection. Studies have shown that not only do KATP channel openers mimic preconditioning, but that blockers abolish the ischemic preconditioning’s protection (7, 129, 305, 362). Further studies have shown that the preconditioning mimicked by adenosine A1 receptor stimulation can also be abolished by glibenclamide, suggesting adenosine receptor activation to be upstream of KATP channel activation (131, 362).

2. The mitoKATP channels

It was initially assumed that the sarcolemmal KATP channel was the end-effector of preconditioning’s protection, and this protection was originally ascribed to shortening of the action potential (for review, see Ref. 130). At the time of these early observations it was not appreciated that cardiomyocytes contained two different types of KATP channels, sarcolemmal (surface KATP) and mitochondrial (mitoKATP), and that each had a distinct phar-
macological profile. However, Garlid et al. (115) and Liu et al. (208) subsequently provided convincing evidence in a recovery of function models and a cardiomyocyte model, respectively, that it was not the surface but the mitoK-ATP channel that was responsible for the protection. Although some data, particularly studies using HMR 1098, a selective sarcolemmal K-ATP channel blocker (342), still suggest a critical role for the sarcolemmal K-ATP channel, most evidence is consistent with the mitochondrial rather than the surface channel as being most important. It should be kept in mind, of course, that virtually all of that theory hinges on pharmacological evidence that could still prove to be flawed.

The K-ATP channel consists of an inward rectifying potassium channel (Kir) in association with a sulfonylurea binding protein (Sur). Several isoforms of each exist, and the channels can assemble in different combinations. The mitoK-ATP channel is thought to have a similar structure to the sarcolemmal K-ATP channel with both a Kir and Sur subunit, although the exact composition of the mito K-ATP channel has not been resolved. However, there are differential pharmacological responses of the cardiac sarcolemmal and mitoK-ATP channels. 5-Hydroxydecanoate (5-HD) inhibits mitoK-ATP channels in the micromolar range, but not sarcolemmal K-ATP channels under any concentration. Dazox, a K-ATP channel opener, has been shown to be 1,000 times more potent in opening mitoK-ATP channels than sarcolemmal K-ATP channels (116).

In addition, dazox-induced cardioprotection has been demonstrated in the micromolar range without any action potential duration (APD) shortening, excluding sarcolemmal K-ATP channel involvement (115). Baines et al. (18) were the first to show that dazox could limit infarct size and that 5-HD could block the anti-infarct effect of both dazox and ischemic preconditioning. Not all data are supportive of the mitoK-ATP role in preconditioning. Recently, Kir6.2 knock-out mice were found to have no functioning sarcolemmal K-ATP channels and also could not be preconditioned despite the fact that their mitoK-ATP were still intact (325).

3. How mitoK-ATP channels could be protective

The mitochondria make ATP by allowing H+ extruded by the electron transport apparatus to reenter along a strong electrochemical gradient through the F1 apparatus. In so doing ADP is phosphorylated to ATP. Opening the K-ATP channel will cause potassium to enter mitochondria along its favorable electrochemical gradient. A potassium/hydrogen exchanger on the inner mitochondrial membrane allows intramitochondrial potassium to exchange for extramitochondrial H+. Entering H+ would theoretically uncouple the mitochondrion because it bypasses F1 and hence reduces ATP production. In actuality, however, the amount of uncoupling resulting from potassium entry is very small (estimated to be ∼5 mV), assumed to be caused by a low density of channels in the inner membrane (114). Terzic’s group has reported the greatest change, which was 10 mV with a baseline of −180 mV in isolated mitochondria (143). These data were confirmed in the intact cell by Minners et al. (227a). The potassium that enters is, however, osmotically active and will cause the matrix to swell.

There are several theories that seek to explain why opening the mitoK-ATP channels should be protective. Terzic and co-workers (144) found that opening mitoK-ATP channels made isolated mitochondria more resistant to Ca2+ entry. Garlid and co-workers (95, 189) suggest that mitochondrial swelling subsequent to potassium entry causes preservation of the functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase on the outer membrane through which ADP traditioned enters the intermembrane space. That juxtaposition effectively keeps ADP out of the intermembrane space and forces the mitochondria to phosphorylate only creatine, which is the most efficient means of transferring energy to the cytoplasm. Of course all of these theories assume that the mitoK-ATP channel is the end-effector of preconditioning’s protection.

4. Trigger role of mitoK-ATP channels

Recent experiments have reexamined the assumption that mitoK-ATP channels are only the end-effectors of protection. Liu et al. (208) introduced a cardiomyocyte model in which FADH fluorescence was monitored. The slight uncoupling with mitoK-ATP channel opening was proposed to slightly oxidize the flavoproteins and increase their fluorescence. These fluorescence studies showed that the effects of both dazox and 5-HD are readily reversible when the drugs are washed out. Yet Ashraf’s group (376) reported that a 5-min pulse of dazox followed by washout put the rat heart into a preconditioned state even though the mitoK-ATP channels should have reclosed when the index ischemia began. Pain et al. (260) repeated the above experiment in the isolated rabbit heart and found that a 5-min pulse of dazox indeed protected the heart against infarction and that the drug could be washed out for as long as 30 min without loss of the protection. Pinacidil, a nonselective K-ATP channel opener, had the same effect. These data suggested that transient opening of mitoK-ATP channels puts the heart into a preconditioned state that continued long after the channel should have closed again.

Pain et al. (260) further studied the timing of channel opening required to protect ischemic hearts. They set out to determine whether mitoK-ATP channel opening was a trigger or mediator of protection. As explained above, triggers act before the index ischemia while mediators act during the index ischemia and therefore must be down-
stream events. If $K_{\text{ATP}}$ opening is the end-effector of preconditioning, then it would be expected to fall into the mediator category. To investigate whether the mito$K_{\text{ATP}}$ channel is a trigger or mediator, both Pain et al. (260) and Wang et al. (373) used isolated rabbit hearts and administration of 5-HD either only during the preconditioning stimulus (early) or only during the index ischemia (late). In both studies, early 5-HD blocked protection supporting a trigger role. Pain et al. (260) were unable to block protection with 5-HD given in the late protocol just before the index ischemia and concluded that mito$K_{\text{ATP}}$ channels were only triggers. Wang et al. (373) however could abort protection if the concentration of 5-HD was increased fourfold over that required to prevent protection in the early protocol. They theorized that a higher concentration may have been required because channel phosphorylation reduced the potency of 5-HD for channel blockade (295). The other possibility, of course, is that the higher concentration of 5-HD introduced a nonspecific effect. Thus, while both investigative groups agree that mito$K_{\text{ATP}}$ channels act as a trigger of preconditioning, Wang et al. (373) suggest that these channels may have a dual role as both triggers and mediators. Further support for mito$K_{\text{ATP}}$ channels as a mediator comes from Gross and Auchampach (129) who infused glibenclamide in dogs between the time of PC and the index ischemia and blocked protection. Gres et al. (126) recently addressed both of these issues in their pig model. In the above study (129), glibenclamide was given right at the onset of reperfusion after the preconditioning ischemia. The reperfusion, of course, would be the critical time for reactive oxygen species formation and requires open mito$K_{\text{ATP}}$ channels. When Gres et al. (126) gave the glibenclamide with the onset of reperfusion, it indeed blocked protection. However, if they delayed administration of the glibenclamide for 5 min but still included it during the index ischemia, protection was unaffected, arguing against any mediator role of K$_{\text{ATP}}$ channels. When they tested a higher concentration of glibenclamide, infarcts were larger but, unfortunately, this concentration of glibenclamide also increased infarct size in nonpreconditioned hearts by a similar increment. On the other hand, infarcts were not increased in nonpreconditioned hearts with the high dose of 5-HD used by Wang et al. (373). Yao et al. (399) also noted in their chick cell model that protection from a PKC activator could be blocked when 5-HD was introduced only during the prolonged simulated ischemia. Thus the current weight of evidence supports both a trigger and a mediator role for the channel. An attractive explanation of this dual role would be a scenario in which channel opening triggers a kinase cascade that feeds back in a positive manner to keep the channel open during the index ischemia.

If mito$K_{\text{ATP}}$ channel opening is an upstream event, then where in the pathway are the signaling kinases located? Ashraf’s group (376) showed that the PKC blocker chelerythrine could block protection from a pulse of diazoxide in the isolated rat heart. While Pain et al. (260) could not show a similar result in the rabbit heart, they were able to block diazoxide’s protection with the tyrosine kinase blocker genistein, indicating that there was at least one tyrosine kinase downstream from mito$K_{\text{ATP}}$ channel opening in the rabbit model. Thus mito$K_{\text{ATP}}$ channel opening protects by activating kinases. This further indicates that mito$K_{\text{ATP}}$ channel opening acts as an upstream link in a signal transduction chain leading to kinase activation. But how could channel opening be a signal?

5. Free radicals and mito$K_{\text{ATP}}$ channels

Steenbergen and co-workers (108) provided the solution to this puzzle. They found that diazoxide’s protection could be blocked by a free radical scavenger, N-acetylcysteine. Their observation was then confirmed by Pain et al. (260) who used the scavenger N-2-(mercaptopyrrolyl)glycine (MPG) in a similar experiment. Yao et al. (400) found that pharmacological preconditioning of chick cardiomyocytes with the muscarinic agonist acetylcholine caused the cells to produce a small burst of free radicals. Yao et al. (407) used the probe 2’7’-dichlorofluorescin diacetate (DCFH) which fluoresces when oxidized by free radicals. This burst could be blocked by myxothiazol (31, 186), indicating that the increased radical production was the result of electron transport within the mitochondria, probably from site III of the electron transport chain where myxothiazol blocks the flow of electrons (for review of free radicals and their cellular origins, see Ref. 98). Furthermore, the burst could be blocked by 5-HD. These observations led Pain et al. (260) to propose a new paradigm incorporating mito$K_{\text{ATP}}$ channels and free radicals in PC’s signaling pathway leading to protection. In this model receptor occupancy leads to mito$K_{\text{ATP}}$ channel opening, which then causes the mitochondria to produce reactive oxygen species (ROS). The free radicals would then activate the downstream kinases that ultimately modulate the end-effector.

Support for the free radical hypothesis was gained by the observation that diazoxide increased free radical production in isolated cardiomyocytes (108) in a human atrial-derived cell line (60) and in vascular smooth muscle cells (186). In all cases the increase in radical production could be blocked by 5-HD. More recently, Oldenburg et al. (254) have shown that exposure of smooth muscle cells to acetylcholine, an agonist known to trigger preconditioning, causes a similar burst of radicals that is dependent on a muscarinic receptor, a pertussis toxin-sensitive G protein, PI 3-kinase, and mito$K_{\text{ATP}}$ channels. Finally, Cohen
et al. (75) found that protection triggered by acetylcholine, bradykinin, norepinephrine, or morphine could be blocked by either 5-HD or a free radical scavenger applied during the trigger phase. The study of Cohen et al. (75) was strong evidence that all of the above receptors couple through the mitoK<sub>ATP</sub> channel/free radical pathway. Interestingly, adenosine was different as neither the K<sub>ATP</sub> blocker nor the scavenger could affect its protection when applied during the trigger phase. Downey and colleagues (75) proposed that adenosine must have had a parallel coupling to the kinases. See Figure 4 for a diagram of the proposed signaling pathways for classical preconditioning.

Qin et al. (282) found that the PI 3-kinase inhibitor wortmannin could block protection from acetylcholine but not from adenosine. Furthermore, it only blocked when the wortmannin bracketed the drug infusion, suggesting that PI 3-kinase acted as a trigger. Unpublished studies reveal that wortmannin does block the acetylcholine-induced burst of ROS in isolated cardiomyocytes and that it is upstream of the mitoK<sub>ATP</sub> channels. Based on that, we would propose that it links the surface receptors to the mitoK<sub>ATP</sub> channel.

The hypothesis that free radicals participate in the trigger signal for preconditioning may answer one of the mysteries of preconditioning: why receptor stimulation during a simple occlusion does not protect the heart. During a prolonged occlusion receptor agonists would be released, populate their receptors, and open the mitoK<sub>ATP</sub> channels. However, the oxygen would be lacking to fuel the burst of ROS so that the signal would die out at that point. Only with reperfusion could ROS be produced so that the signal transduction pathway could be completed. In support of that observation, several investigators found that a period of total occlusion protected both pig (303) and rabbit (107) hearts from a subsequent period of low-flow ischemia. Receptor-mediated opening of mitoK<sub>ATP</sub> channels during the total occlusion period would result in ROS formation during the low-flow period when oxygen was again available. Not all investigators agree on when the ROS are formed, however. Becker et al. (31) report significant ROS formation during simulated ischemia in chick cardiomyocytes. Whether there is enough residual oxygen available during a preconditioning ischemia to make the ROS signal without reperfusion is currently unknown.

6. Prostaglandins

Several studies have looked at prostaglandin pathways in ischemic preconditioning. Murphy et al. (237) found that preconditioning’s preservation of postischemic function in the rat model could be blocked by a 12-lipoxygenase inhibitor. More recently, Gres et al. (127) found that indomethacin, a cyclooxygenase blocker, could abolish protection from a weak but not a strong preconditioning protocol in open-chest pigs. Whether cyclooxygenase was acting in the trigger or mediator phase was not investigated. Indomethacin could not block preconditioning in rabbits (205), and cyclooxygenase 1 and 2 knock-out mice could still be preconditioned (54). Clearly the role of prostaglandin pathways in preconditioning warrants further investigation.
D. Possible End-Effectors

1. Metabolic effects

The end-effector of preconditioning has been amazingly elusive. After more than a decade and a half of intensive research, the actual mechanism whereby the cell is protected against lethal injury is an enigma. There have been many theories over the years. The oldest theory was that of Murry et al. (241), who suggested an improved energy balance in the preconditioned ischemic myocardium. They proposed that perhaps the mitochondrial ATP-ase activity had been inhibited in these hearts. Although energetics are improved in preconditioned hearts, there is convincing evidence that that may not be the mechanism. In some protocols the favorable energy balance during ischemia may not be great enough to overcome the initial deficit caused by the preconditioning ischemia itself. Ko-lacassides et al. (182) found that their ischemically pre-conditioned rat hearts actually went into contracture earlier and had consistently lower ATP levels during the index ischemia despite obvious protection. The preconditioned hearts recovered from contracture during reperfusion, whereas the nonpreconditioned hearts did not.

2. The mitoK_ATP channel

The mitoK_ATP channel remains a viable candidate. Particularly in light of compelling evidence that it must reopen during the index ischemia, the likely time where the end-effector is exerting its effect. How the opening would protect the ischemic heart is not so clear as it will cause a slight uncoupling of the mitochondria and swelling (for a review, see Ref. 114); neither effect would be expected to protect. Terzic’s group (144) finds that K_ATP channel openers make the mitochondria resistant to calcium overload. Perhaps opening mitoK_ATP channels prevent opening of the mitochondrial transition pore (156) during deep ischemia. Indeed, in a paper recently published, Hausenloy et al. (137) describes a new paradigm for preconditioning in which opening the mitoK_ATP channel could act to prevent opening of the mitochondrial transition pore described below.

3. Mitochondrial permeability transition pore

Halestrap et al. (135) proposed that ROS generation at reperfusion plus calcium entering the cell could cause adenine nucleotide translocase (ANT) to open a large-diameter pore within the mitochondrial membranes. The pore formation involves the binding of mitochondrial cyclophilsins to the ANT and can be prevented by treating the hearts with cyclosporin A which binds the cyclophilsins. The transition pore disrupts mitochondrial function and allows foreign substances into the matrix, which effectively destroys the mitochondria. It has long been recog-nized that pretreatment with cyclosporin A is cardioprotective. However, it was not known whether the protection derived from its inhibitory effect on phosphatases (378) or its inhibition of transition pore opening (128).

Yellon’s group (137) recently proposed that ischemic preconditioning might act to prevent opening of this pore. They demonstrated that protection from either ischemic preconditioning, diazoxide or an adenosine agonist could be blocked by actractyloside, an opener of the transition pore. Actractyloside had no effect on infarct size in non-preconditioned hearts. Diazoxide also inhibited calcium-induced pore opening in isolated mitochondria. This is persuasive evidence supporting the transition pore as the end-effector of preconditioning. Arguing against inhibition of the transition pore as an end-effector of preconditioning is a recent study by Garlid’s group (95). In that study mitochondria isolated from ischemic hearts showed no difference in state 2 respiration compared with those from nonischemic hearts. That would not be expected if a transition pore had opened in these mitochondria. In that study the outer mitochondrial membranes did show an increased permeability to proteins which preconditioning prevented, however.

4. The sodium/hydrogen exchanger

Xaio and Allen (386) have suggested that the sodium/proton exchanger might be the end-effector of ischemic preconditioning and that its inhibition might lead to protection of the ischemic heart. Certainly pharmacological inhibition of the exchanger is one of the most potent protectors of the ischemic heart yet discovered (165). Xaio and Allen noted that the sodium/proton exchanger appeared to be blocked at reperfusion only when rat hearts had been preconditioned. Addition of HOE 642, a highly selective blocker of the sodium/proton exchanger, shortly before reperfusion to a nonpreconditioned heart preserved postischemic function by an amount equal to that achieved by ischemic preconditioning. Hoe 642 had no additive effect when combined with ischemic preconditioning, further suggesting a common mechanism. Similarly, 5-(N-ethyl-N-isopropyl)amiloride, another blocker of the sodium/proton exchanger, limited infarct size in rabbits by an amount equal to that of preconditioning, and it could not augment preconditioning’s anti-infarct effect (293). Neither kinase inhibitors nor 5-HD could abolish amiloride’s protection, suggesting that the sodium/proton exchanger must be protective as an end-effector. Probably the most serious criticism of the hypothesis is the PKC, a key step in preconditioning acts to activate rather than inhibit the exchanger (164).

5. Osmotic swelling

Cells are in osmotic equilibrium and cannot tolerate an osmotic imbalance. Osmotic balance is maintained by
matching the osmotic pull of proteins and nucleotides within the cell primarily by sodium outside the cell. Because the conductance of the sarcolemma to sodium is very low, extracellular sodium is an efficient osmolyte. That is why virtually every cell type excludes sodium and maintains a negative resting membrane potential. During ischemia ATP is broken down to AMP and two inorganic phosphates, thus tripling the osmotic pull of the nucleotides. Similarly, failure of the sodium-potassium pumps leads to sodium leak into the cell and thus a collapse of the vital sodium gradient. Each millimolar increase in osmolyte concentration exerts an additional 19 mmHg transmembrane pressure. Indeed, Jennings and co-workers (381) proposed that osmotic swelling was the cause of membrane failure and cell death in reperfused myocardium in 1974. During ischemia, movement of water into the cell concentrates the extracellular sodium in 1974. During ischemia, movement of water into the cell concentrates the extracellular fluid and thus limits swelling. Upon reperfusion however, the extracellular fluid is replaced with isotonic fluid and explosive swelling ensues.

Preconditioning makes cardiomyocytes very resistant to membrane failure when they are challenged with hypotonic media (12). In ischemically preconditioned rat (109) and pig (292) hearts, the extent of myocardial edema formation, along with infarct size, is reduced. Alterations in channels involved in cell volume regulation therefore might be involved in the cardioprotection achieved by ischemic preconditioning. Chloride channels are involved in moment-to-moment volume regulation, and Diaz et al. (93) hypothesized from experiments in rabbit cardiomyocytes and isolated hearts that opening of swelling-induced chloride channels was responsible for the protection achieved by ischemic preconditioning. However, their electrophysiological and infarct size data could not be confirmed (140). There are also organic osmolytes, such as taurine or sorbitol, which are involved in ischemia/reperfusion damage. In anesthetized rats, taurine depletion indeed reduces infarct size following ischemia/reperfusion (14). Preconditioned hearts are more resistant to hypotonic media (12). This theory ties in with the p38 MAPK hypothesis of preconditioning nicely, since activation of p38 through MAPKAPK2 causes phosphorylation of the small heat shock proteins hsp27 and its smaller isoform, αβ-crystallin, which in turn causes actin filament assembly in the cytoskeleton (132) and is very protective in other cell types (146). As noted above, the evidence supporting p38 MAPK is controversial. Nevertheless, Eaton et al. (100) did correlate phosphorylation of αβ-crystallin with protection from ischemic preconditioning in rat hearts. Holly et al. (141) reported a translocation of hsp27 and αβ-crystallin to the cytoskeleton after preconditioning. Dana et al. (87) also correlated hsp27 phosphorylation with preconditioning’s protection in a SWOP model of preconditioning. On the other hand, Armstrong et al. (13) could not correlate phosphorylation of hsp27 with protection in preconditioned rabbit cardiomyocytes.

7. Apoptosis

Another possible tie in with the MAPKs would be an antiapoptotic effect of preconditioning. Gottlieb et al. (123) proposed that ischemia/reperfusion triggers programmed cell death in the heart and that much of the tissue loss is a direct result of triggered apoptosis (for a review of apoptosis in infarction, see Ref. 10). Preconditioning has been reported to lower the levels of proapoptotic BAX protein in the heart (243) and prevent caspase activation during ischemia/reperfusion (276). Caspase inhibitors are reported to mimic preconditioning by limiting infarct size in some studies (142, 231, 401) but not in others (251). Ohno et al. (250a) pointed out that the commonly used marker for apoptosis (nick end-labeling by TUNEL method) was confined to cells which already showed lethal oncotic (swelling) injury and thus may not have been the actual cause of cell death. Another problem with the apoptosis hypothesis is that it is difficult to kill a cell quickly by disabling its nucleus. Hearts can live for hours in the presence of complete protein synthesis inhibition (340). Yet, cell death is complete after only 2 h of reperfusion. Thus it has not been resolved whether apoptosis is important in preconditioning or whether the reduced apoptosis seen in preconditioned heart is secondary to protection from other mechanisms.
8. Gap junctions

An intriguing hypothesis is that preconditioning may act to close gap junctions in the heart. It has been known for a long time that infarcts tend to be confluent, suggesting that necrosis spreads from one cell to the next. This spread could occur through gap junctions, the low-resistance channels between adjacent heart muscle cells. Transgenic mice deficient in connxin43 (a major component of cardiac gap junctions) could no longer be protected by preconditioning (306). Heptanol, a closer of gap junctions, also blocked the protective effect of preconditioning in isolated mouse hearts (199). The above studies suggest that opening of gap junctions rather than closing them is the protective step. Conversely, heptanol appeared to mimic preconditioning in isolated rabbit hearts (290). For a comprehensive review on this subject, see Reference 113.

9. Free radicals

During the 1980s it was proposed that free radicals might contribute to cell death in the reperfused hearts. Although the early animal data were encouraging, there was difficulty in repeating those studies as the infarct size models became more robust (96, 177, 285). To absolutely prove that free radicals contribute to cell death in ischemia reperfusion injury, one would have to unambiguously show that a free radical scavenger introduced at reperfusion can reduce infarct size. That has never been done to the satisfaction of all. Nevertheless, the hypothesis persists since it has never been disproven either. Accordingly, preconditioning may act to reduce the free radical production at reperfusion. Turrens et al. (356) measured antioxidants in preconditioned hearts and found no difference from that in nonpreconditioned hearts. On the other hand, Steeves et al. (319) did report an increase in antioxidants in preconditioned rat hearts. Vanden Hoek et al. (359) using a cell model did see less radical production at reperfusion in preconditioned chick cardiomyocytes, supporting a free radical hypothesis. Arguing against the possibility that reduced radical production could have simply been the result of less injury in those cells was the observation that scavengers mimicked the preconditioning. Also in support of a free radical hypothesis is the observation that superoxide dismutase is increased in hearts with the second window preconditioning (see sect. nD2).

10. Tumor necrosis factor-α

There is compelling evidence that the toxic autacoids tumor necrosis factor-α (TNF-α) may play a role in preconditioning. TNF-α was originally proposed to contribute to ischemic cell death. Meldrum et al. (223) found that even crystalloid-perfused rat hearts made TNF-α during ischemia and that it was reduced in hearts preconditioned with adenosine. It has also been reported that ischemic preconditioning reduced TNF-α in situ rabbit hearts during ischemia. The protected hearts showed an increase in TNF-α inhibitory serum activity which could account for the reduced TNF-α (38). The same effect on TNF-α was seen in a SWOP model where rabbits were exposed to lipopolysaccharide 3 days before the ischemic insult (38). Interestingly, TNF-α has also been proposed to act as a trigger for preconditioning protection. Yamashita et al. (393) found that antibodies against TNF-α could abolish the induction of both Mn-SOD and SWOP against infarction from ischemic preconditioning in rat hearts. Most recently, Smith et al. (316) found that TNF-α knock-out mice could not be acutely preconditioned by ischemia but could still be protected with either adenosine or diazoxide. Furthermore, a transient exposure to exogenous TNF-α put the wild hearts into a preconditioned state but inexplicably could not protect the TNF-α knock-outs.

E. Summary of Classic Preconditioning

Although much has been found concerning the cellular mechanisms of ischemic preconditioning, there are still glaring gaps in our knowledge. We can be certain that surface receptors, KATP, free radicals, and PKC all play pivotal roles in the signaling of the protective signals. Beyond that we find very conflicting reports as to the exact details of these signaling pathways. It is even not certain whether the KATP channel is on the surface or the mitochondria. Whether KATP merely plays a signal transduction role or whether it is the end-effector still remains to be established. Although a number of other end-effectors have been proposed such as the permeability transition pore, sodium/hydrogen exchanger, apoptosis, gap junctions, etc., there is insufficient data available to support any one to the exclusion of the others. The difficulty is that all of these studies have led us to fields where the technology is clearly lacking. Until novel techniques are developed which allow us to work with things like the transition pore, mitoKATP, or gap junctions, these hypotheses will be difficult to prove.

IV. NATURAL HISTORY OF THE SECOND WINDOW OF PROTECTION

A. What is SWOP?

In 1993 it was reported that if the intervening period of reperfusion between the preconditioning and the test ischemia is extended to 24 h, a delayed phase of myocardial protection is induced (188, 213), which although not as powerful as the early phase, is more prolonged and
lasts up to 72 h (26). This delayed phase of resistance to ischemic injury as mentioned above has been termed by Yellon’s group as the second window of protection or SWOP (213), distinguishing it from early or “classic” preconditioning. SWOP has variously been referred to as “delayed” or “late” preconditioning as well. It is likely that these two forms of adaptation have different underlying mechanisms, although they share the same trigger, transient ischemia.

Additional studies in rabbits (397) and rats (394) have since confirmed these original findings. More recent evidence suggests that in addition to enhanced tolerance to lethal ischemic injury, delayed preconditioning confers protection against other end points of ischemia-reperfusion injury including ischemia and reperfusion-induced ventricular arrhythmias (365) and posts ischemic myocardial dysfunction (stunning) (45, 323). The delayed protective effects of preconditioning have also been demonstrated in vitro, using isolated cardiomyocytes subjected to simulated ischemia or hypoxia (82, 396).

Similar to classical preconditioning, SWOP can be subdivided into the triggers that exert their actions during the preconditioning ischemic period and mediators that act during the subsequent index ischemia. The prolonged interval between the preconditioning event and its renewed protection a day later allows for the possibility of new protein synthesis, posttranslational protein modification, and a change in the compartmentalization of existing proteins (404).

B. Triggers

1. Adenosine

The SWOP can be stimulated by a spectrum of non-pharmacological and pharmacological stimuli. The former include ischemia, heat stress, rapid ventricular pacing, and exercise, while the latter include adenosine receptor agonists, bradykinin, opioid agonists, NO donors, cytokines, ROS, and endotoxin (e.g., monophosphoryl lipid A). Interestingly, the triggers appear mostly identical to those of acute ischemic preconditioning, although there appears to be significant interspecies variation with respect to the relative importance of each trigger: adenosine, NO, ROS (324), and opioid receptor agonists (109) are all important.

Yellon and colleagues (27) were the first to show adenosine receptor involvement in the delayed phase of myocardial protection 24 h after ischemic preconditioning. They went on to demonstrate the temporal nature of this delayed effect with the adenosine A1 receptor agonist, which lasted up to 72 h (29), was very similar to that seen when preconditioning was induced with ischemia (26). Studies using receptor stimulation to produce a delayed protective effect have also been seen in other species including the rat (86).

Interestingly, adenosine acting on A1 and possibly A3 receptors (327) was able to trigger a SWOP against infarction but not against myocardial stunning (16). Tsuchida et al. (355) undertook studies in which they tried to maintain the heart in a classic preconditioning state by giving a continuous infusion of 2-chloro-N6-cyclopentyladenosine (CCPA) but were unable to demonstrate any protection which they attributed to a desensitization of the A1 receptor. Dana et al. (84) however tried to overcome this potential problem by giving repeated administration of CCPA at 48-h intervals for a 10-day period and were able to maintain a continuous protective effect against infarction over the entire time without any evidence of adenosine A1 receptor downregulation.

2. NO

NO has been shown to play an important but as yet undefined role in delayed preconditioning against both MI and myocardial stunning (329). Indeed, NO generated in ischemic tissue has been proposed to act as both a trigger as well as distal mediator of SWOP by Bolli and colleagues (44, 46). This group demonstrated that nitro-L-arginine (L-NA) administered 24 h after an ischemic preconditioning protocol. They clearly showed that NOS inhibition blocks the SWOP (328), and pretreatment with NO donors in the absence of ischemia induces a similar delayed protective effect (133). Indeed, Bolli et al. (46) were the first to come up with the so-called NO hypothesis of late preconditioning. As mentioned above they propose that NO has a bifunctional role in delayed preconditioning. eNOS triggers the release of NO in association with the preconditioning stimulus, and inducible NO synthase (iNOS) then mediates the formation of NO 24–72 h later to protect against subsequent ischemia. This hypothesis is supported by direct measurements of NOS activity that show a biphasic regulatory pattern (388).

Bolli’s group (47, 328) further investigated the role of NO in delayed preconditioning using the NO blocker N\(^{\text{G}}\)-nitro-L-arginine (l-NA) administered 24 h after an ischemic preconditioning protocol. They clearly showed that it blocked the preconditioning protection against infarction (328) as well as against stunning (47) in conscious rabbits. A number of studies using the mouse heart with targeted disruption of the iNOS gene have demonstrated that iNOS is pivotal to the mediation of the SWOP. In this regard Bolli and colleagues (133) using ischemia as a preconditioning trigger were unable to demonstrate any protection in the in vivo iNOS knock-out mouse. Furthermore, Kukreja and colleagues (385) using an in vitro iNOS knock-out mouse model were similarly no longer able to protect with the endotoxin derivative monophosphoryl lipid A, a pharmacological trigger of SWOP. Interestingly, Yellons’ group has suggested that although NO is essential to the mediation of delayed protection, the source of this
NO need not necessarily be derived from iNOS. This was first indicated in pharmacological studies using the aden- osine A1 receptor agonist CCPA in which they were unable to abolish the second window using the specific iNOS inhibitors L-N^6-(1-iminoethyl)-lysine (L-NIL) and amino- guanidine in rabbit (85). This contradictory result was investigated in more detail by Bell et al. (35) in the iNOS knock-out mouse. In these studies they found that CCPA could elicit protection in iNOS knock-out mice concomit- ant with a significant upregulation of the eNOS protein. Moreover, the protection was abrogated by a nonselective NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). These results suggest that in certain circumstances eNOS can masquerade as iNOS to mediate protection. Further support for this comes from data showing enhanced NO in the myocardium following brief episodes of ischemia/ reperfusion, which is thought likely to be due to eNOS since SWOP can be blocked by nonspecific NOS inhibition, but not by the selective iNOS inhibitors aminoguanidine and S-methylisothiourea (47).

Because of the evidence supporting NO as a trigger of SWOP, it has also been examined in classical precondition- ing. Isolated rabbit hearts continued to be protected by ischemic preconditioning in the presence of L-NAME, strongly arguing against such a trigger role (247). Interestingly, however, an NO donor could trigger a precondi- tioned state in that study.

3. Bradykinin

We know that many of the triggers of early precon- ditioning are also involved in the second window, and in this regard bradykinin is no different. This has been ob- served in both the rat and rabbit heart (101, 184) In the former study, bradykinin administered to rats resulted in protection in infarction 24 h later. This effect was abolished if the rats were treated with L-NAME to inhibit NOS before the bradykinin administration. That is in contrast to studies in classical preconditioning where bradykinin will also precondition the rabbit heart, but in that case L-NAME had no effect against the protection (122). The rabbits were either preconditioned with sublethal ischemia in the presence of the bradykinin B2 receptor blocker HOE 140 or given bradykinin directly. When the hearts were examined 24 h later, bradykinin was seen to be equal to preconditioning in terms of myocardial pro- tection. In addition, the HOE 140 completely abrogated the protection seen following as a consequence of the preconditioning stimulus. Both studies clearly demonstrate a role for the B2 receptor in delayed precondition- ing.

Indirect evidence for the importance of bradykinin, as a trigger of delayed preconditioning, also comes from studies in pigs by Yellon’s group (155). Here they demon- strated that two 2-min periods of ischemia, caused by balloon inflation in a coronary branch of pig hearts, were insufficient to induce delayed protection. However, when they administered an ACE inhibitor, concomitantly with the subthreshold preconditioning stimulus, they could detect a fully protective response 24 h later. Since it is known that ACE inhibition prevents the degradation of bradykinin, these results add further to the evidence of the importance of this peptide as a preconditioning mimetic.

4. Opioids

Although the evidence for a role for opioids in de- layed preconditioning is still limited, a number of recent studies have shown that opioids can act as triggers for this phenomenon. In this regard Gross and colleagues (109) were the first to show that a δ-opioid agonist could induce a delayed cardioprotection in the rat. This protection was associated with mitoK\textsubscript{ATP} channel activation as the mitochondrial blocker 5-HD and glibenclamide were shown to abolish the effect of the δ-opioid agonist TAN-67. Furthermore, this study also demonstrated the tempo- ral nature of the opioid agonist in that the protection they observed was seen after 24- and 48-h treatment but was lost by 72 h. In attempting to further investigate the mechanism, this group has shown, using the rat, that this delayed effect, as with early preconditioning, appears to involve both ERK and p38 MAPK as integral components of the cardioprotection (110). This was followed by studies in their laboratory where they used a specific δ-opioid agonist to demonstrate delayed cardioprotection via a free radical mechanism that was only partially due to opioid receptor stimulation (265). These ongoing studies are impor- tant as the use of agents, such as δ-opioid agonists, may be of clinical relevance in the setting of patients with acute coronary symptoms who are at risk of MI.

5. Other proposed triggers

It is of interest to note that prostanoids have been shown to produce a delayed form of cardioprotection for many years. Indeed, Szekeres et al. (326) described how 7-oxoprostaglandin could induce a “delayed and long last- ing” protection to the heart over a decade ago. This was long before the second window or delayed precondition- ing effect was first described (213).

Similarly, the administration of catecholamines, such as norepinephrine, has also been shown to produce de- layed cardioprotection in rats (225). In an earlier study the same group (224) demonstrated how norepinephrine could induce hsp70, which in turn was associated with delayed cardioprotection in rat. Catecholamines could either trigger the SWOP directly through the α\textsubscript{1}-receptors or they might drive the heart into a demand ischemia where other factors such as adenosine and bradykinin could be the actual triggers of the preconditioned state.
C. Mediators: Signal Transduction Pathways

1. PKC

Multiple signal cascades are activated in response to transient reperfusion/reperfusion. PKC, protein tyrosine kinase, and the MAPKs including the activation of various transcription factors that are linked to the expression of the cardioprotective genes such as nuclear factor-kappa B (NF-κB) are all thought to be part of the signaling mechanism of SWOP. For a comprehensive review, see Reference 19.

The first to demonstrate a role for PKC in the SWOP was Yamashita et al. (396) who demonstrated that the PKC inhibitor staurosporine could prevent the acquisition of this phenomenon in hypoxically preconditioned myocytes. With regard to MI however, the first evidence indicating a role for PKC in the mechanism of delayed protection was seen in the rabbit model (25). Subsequently, Qui et al. (283) demonstrated that, as in classic preconditioning, PKC is also an essential mediator of the second window. This was followed by studies in which a PKC agonist, diocanoyl-sn-glycerol, was observed to cause a significant reduction in infarct size in rabbit hearts 24 h after administration (28). With regard to other end points of injury, inhibition of PKC-ε translocation, using chelerythrine, completely blocked the delayed preconditioning against stunning (283). This PKC activation was seen to be NO dependent and could be blocked by pretreatment with the nonselective NOS inhibitor L-NA (271). Indeed, there have been a number of studies by Ping and colleagues (272–275) demonstrating the importance of PKC in delayed preconditioning against both stunning and infarction. With regard to translocation of PKC-ε to the membrane, this has also been shown to occur in dog hearts following rapid cardiac pacing, which is associated with protection against arrhythmias (383).

2. Tyrosine kinases and MAPKs

Imagawa et al. (151) demonstrated a role for protein tyrosine kinase in delayed preconditioning. They showed that the protein tyrosine kinase inhibitor genistein abolished the protection seen 48 h after ischemically preconditioning the rabbit heart. Recent studies have also focused attention on two members of the Src family of protein tyrosine kinases, namely, the Src and Lck. Protein tyrosine kinase activation is blocked by the PKC inhibitor chelerythrine, indicating the downstream position of the protein tyrosine kinase in respect of PKC (275). Lavendustin A (LD-A) has also been shown to block protein tyrosine kinase activation and abolish the delayed preconditioning against myocardial stunning (91). Furthermore, Yellon and colleagues demonstrated that LD-A blocked the adenosine A1 receptor agonist (CCPA)-induced SWOP using infarct size as the end point (87), although the protection of heat shock-induced SWOP could not be inhibited using genistein (163).

Maulik et al. (220) demonstrated that ischemic preconditioning could trigger activation of MAPK in rat hearts (220). Unfortunately, the role of MAPKs in SWOP remains unresolved, primarily due to variations in experimental protocols. All three MAPK subfamilies, namely, the p42/p44 MAPK, the p38 MAPK, and the JNKs are induced by a SWOP protocol in a rabbit model. In that study infarct size measurement and MAPK activation were studied in different animals (272, 273). However, in a pig model, no correlation between infarct size reduction and MAPK activation (measured within the same animal) could be demonstrated (33). Furthermore, using isolated myocytes, Heads and colleagues (235) have shown that metabolic inhibition can induce delayed preconditioning, an effect which is PKC dependent but independent of MAPK activation. Thus further research in this area is needed to confirm the precise role of MAPKs in this form of preconditioning.

The transcriptional regulator NF-κB is responsible for the modulation of many genes. It may well be a common downstream pathway through which multiple signals generated by ischemia (NO, ROS, PKC, and protein tyrosine kinases) are able to initiate cardiac gene expression. Both ischemic preconditioning and NO donors are able to induce NF-κB activation (387). Conversely, Xuan et al. (387) were able to abrogate SWOP by the direct inhibition of NF-κB. At the same time activation of NF-κB following an ischemic preconditioning protocol was disrupted by pretreating with known blockers of SWOP, including the NOS inhibitor l-NA, mercaptopropionyl glycine (an antioxidant), chelerythrine, and LD-A. It is possible that other stress-responsive transcription factors, e.g., activating protein 1, may play a similar role in focusing the SWOP effect. It should also be noted however that NF-κB, as well as other transcription factors, can also be activated by prolonged ischemia (irrespective of a preconditioning stimulus), which in itself may result in inflammatory injury (63). See Figure 5 for a diagram of the proposed signaling pathways involved in SWOP.

D. Possible End-Effectors

1. Heat stress proteins

The first studies investigating the existence of a SWOP were based on studies which demonstrated that 24 h after heat shock, a significant protection against subsequent MI could be observed (for review, see Ref. 406). This protection was linked to the upregulation of the 72-kDa inducible heat stress protein (hsp72i). It was known that ischemia, like heat shock, was also capable of inducing the appearance of a cardiac mRNA coding for proteins similar to hsp72i (94) as well as causing the rapid
and direct expression of heat shock proteins in rabbit heart after brief ischemia (180). It was, therefore, hypothesized that it should also be possible to see significant protection 24 h after ischemic preconditioning. This indeed proved to be the case, and it was subsequently demonstrated in rabbits that 24 h after an ischemic preconditioning stimulus, there was an increase in hsp72 as well as a significant reduction in MI (213). This group provided further evidence of protection using transgenic mice overexpressing hsp72 (214). Subsequent studies in which the human hsp72 gene was transfected directly into cardiac-derived cell lines (138) and myotubes (42) also demonstrated protection against subsequent hypoxic injury. However, it must be noted that some studies have not supported this hypothesis, with some reporting no hsp72 induction in rabbits preconditioned with ischemia (333). Rats exposed to ischemic preconditioning have also not had sufficient elevation of hsp72 to correlate with protection against infarction (281). The smaller heat shock proteins, i.e., hsp27 and αB-crystallin, have also been implicated in the second window and could act via the cytoskeletal mechanism described above (see sect. vD6). Interestingly, although it has been shown that pharmacological induction of delayed preconditioning can occur using an adenosine A1 receptor agonist, the resulting protection has been linked to the smaller hsp27 rather than hsp72 (87). Thus the relative importance of the heat stress proteins in delayed preconditioning are as yet unresolved and more work needs to be undertaken to define their precise role.

2. Antioxidant enzyme systems

The antioxidant enzyme systems are another group of proteins that show either increased transcription or functional upregulation. The three main enzyme systems in the heart are superoxide dismutase (SOD), catalase, and glutathione peroxidase. The SODs are a family of metalloenzymes responsible for the dismutation of \( \text{O}_2^- \). Hoshida et al. (145) were the first to demonstrate an increase in MnSOD activity (mitochondrial origin) in ischemic versus nonischemic myocardium immediately fol-
following preconditioning in the canine heart. This increased activity was lost by 3 h and reappeared at 24 h after ischemic preconditioning. MnSOD content was increased at 12 and 24 h after preconditioning (indicating de novo protein synthesis), but not at less than 6 h. No changes in Cu/ZnSOD activity or protein content (cytoplasm origin) were observed. Kuzuwa et al. (188), from the same group, were the first to demonstrate, in dog myocardium, that following short periods of ischemia there was a delayed protective effect against a subsequent lethal ischemic insult 24 h later, an effect which they attribute to the upregulation of antioxidant enzymes such as MnSOD (145). They subsequently demonstrated the same effect in rat cardiac myocytes (396) and more recently in the rat in vivo (391). In this latter study they demonstrated that antisense oligonucleotides directed against manganese SOD abolished the protection in infarction in vivo, strongly implicating this antioxidant in delayed preconditioning. Furthermore, heat shock also induced MnSOD activity in rat cardiac myocytes, and tolerance to hypoxia was abrogated by the administration of MnSOD antisense oligodeoxynucleotides (hsp72 expression was unaffected) (380). Dana et al. (86) showed that MnSOD activity was increased 24 h after treatment with the adenosine A1 receptor agonist CCPA, which was then attenuated by prior administration of PKC and protein tyrosine kinase inhibitors (86). In vivo administration of antisense oligodeoxynucleotides to MnSOD has now been reported to block ischemia-induced (391), heat stress-induced (390), exercise-induced (392), and CCPA-induced (86) SWOP. However, it must be mentioned that not all studies have found upregulation of antioxidant defenses in the delayed preconditioning. In conscious pigs subjected to ischemic preconditioning, Tang et al. (335) could detect no change in MnSOD, Cu/ZnSOD, catalase, glutathione peroxidase, or glutathione reductase activity 24 h after the preconditioning stimulus.

3. Cyclooxygenase

A recent development from Bolli’s laboratory (312) proposes that cyclooxygenase (COX)-2 acts in the genesis of SWOP. Increased COX-2 expression (with a concomitant rise in prostaglandin E2, I2, and F2α) was found 24 h after an ischemic preconditioning protocol in rabbits. The cardioprotective effects of the SWOP were blocked following the administration of two unrelated COX-2-selective inhibitors at 24 h after the ischemic preconditioning protocol. In addition, a COX-2 inhibitor was also found to abolish delayed preconditioning against infarction in the mouse heart (124). Confirmation of the potential importance of cyclooxygenase has recently been shown using COX-1 and -2 knock-out mice in which a decrease in postischemic recovery of left ventricular diastolic pressure was observed (55). How COX-2 mediates protection is not clear; however, recent evidence appears to link inducible NOS to the modulation of COX-2 activity in rabbit hearts (313). If that proves to be true, it would reveal yet another important role for NO in this protective phenomenon. In contrast to COX-2-induced late preconditioning however, the mechanisms associated with adenosine A1 and A3 receptor-induced delayed protection appear independent of COX-2 (181). Thus further work is needed to confirm all the above findings especially since COX-2 has previously been thought of as detrimental to cellular survival (361).

4. The mitoKATP channel

As with classic preconditioning, attention has focused on the importance of these channels in delayed preconditioning, specifically the role of the KATP channels of the mitochondrial inner membrane rather than those located on the sarcolemma. Pharmacological evidence with inhibitors of KATP channel opening suggests that these channels play a key role in conferring protection by delayed preconditioning. While glibenclamide is an inhibitor of both channels, 5-HD inhibits mitochondrial channels selectively. Bernardo et al. (39) reported that delayed preconditioning in rabbit was abolished when either glibenclamide or 5-HD was administered just before the index ischemic insult, suggesting a critical role of KATP channel opening. This was concluded to be due to the sarcolemmal KATP channel, since differences in monophasic action potential duration were seen with both drugs. This observation does not sit easily with the fact that most workers find that 5-HD has no effect on sarcolemmal KATP channel currents. Nevertheless, the study tends to support the idea that enhancement of some aspect of KATP channel function is a pivotal event in mediating delayed preconditioning. Takano et al. (330) have recently confirmed that 5-HD given before index ischemia abolished delayed preconditioning against infarction but did not modify the delayed antistunning effect of preconditioning.

Although it is unproven at present, it is possible that upregulation of some protein associated with the KATP channel alters its opening characteristics, and this determines the protection afforded by delayed preconditioning. Another possibility is that elevated NO levels, generated as a result of iNOS induction, modify KATP channel activity. There is evidence that NO stimulates cGMP-dependent protein kinase (PKG), which in turn can activate KATP channels. Whatever the precise molecular mechanism, the pharmacological evidence with 5-HD would tend to favor involvement of mitochondrial rather than sarcolemmal KATP channels (207).

It is now known that delayed cardioprotection can be induced by a variety of nonischemic stimuli, including adenosine A1 agonists (30), δ-opioid agonists (109), heat stress (267), and monophosphoryl lipid A (222), and that protection from all these stimuli are sensitive to inhibition by either glibenclamide or 5-HD given just before the index
ischemic event. Such evidence points to an intriguing common distal mechanism of action in delayed cardioprotection studies. A further interesting development is that mitoK_ATP channel openers such as diazoxide are able to induce pharmacological delayed preconditioning (250, 330). The possible mechanisms by which mitoK_ATP channel opening can act as a signal transduction agent to activate kinases are discussed in section IV.C.4. Indeed, Takashi et al. (331) have reported that diazoxide can trigger a delayed protection via a PKC-dependent mechanism.

5. NO

As mentioned above, NO has been proposed as both a trigger as well as end-effector of SWOP (46). Initially Vegh et al. (366) demonstrated that delayed preconditioning against arrhythmias, induced by rapid pacing, was due to the inhibition of NOS and cyclooxygenase induction using the nonspecific agent dexamethasone. They proposed that this effect was due to an upregulation of iNOS (366). Bollis’ group then provided strong evidence using pharmacological studies (328) as well as studies in iNOS knock-out mice (134) which demonstrated a role for iNOS in mediating delayed preconditioning against myocardial infarction in rabbits. Yellon’s group (151) had earlier demonstrated that dexamethasone given to rabbits before ischemic preconditioning abrogated protection against infarction 48 h later. They also showed that aminoguanidine, a selective inhibitor of iNOS activity, before the sustained ischemic insult, also abolished the protection. It is important to note however that the upregulation of iNOS with subsequent enhanced NOS production does not appear to occur in all forms of delayed preconditioning. Although one can observe that SWOP is abolished by inhibitors of iNOS (151, 328) in addition to being absent in iNOS knock-out mice (134), the role of iNOS in delayed pharmacological preconditioning, using adenosine A1 receptor agonists, is not clear. Some studies using such agonists have been shown to induce delayed protection against infarction, which is not abrogated by iNOS inhibitors (85) and can be demonstrated in animals in which the iNOS gene has been disrupted (35). As mentioned earlier, however, at least one other study reports the opposite of this (413). We believe that it is difficult at this stage to draw conclusions as to the role of NOS with relation to it acting as either a trigger or mediator (or both) of delayed preconditioning (this section should be read in conjunction with section IV.B2).

V. PRECONDITIONING HUMAN MYOCARDIUM

The question that emerges from the wealth of animal-based evidence indicating the importance and power of the preconditioning phenomenon is whether this form of protection can be seen in humans. Furthermore, if it can, could it be exploited to design therapeutic strategies to prophylactically protect the human heart against infarction?

The obvious ethical restrictions associated with studying ischemic preconditioning in humans have been ingeniously circumvented by studying surrogate end points of reperfusion-reperfusion injury in experiments using human ventricular myocytes, and atrial trabeculae studies and in patients with naturally occurring ischemic syndromes. In addition, studies of patients undergoing planned procedures that involve brief periods of ischemia such as coronary angioplasty (PTCA) and coronary artery bypass surgery (CABG) have also been used to demonstrate that preconditioning can occur in patients at risk of impending MI. In the following sections we review the available evidence.

A. In Vitro Studies

1. Human cell preparations

Ikonomidis et al. (148) were the first to demonstrate preconditioning in human ventricular myocytes in cell culture using trypan blue exclusion and metabolic end points of injury. This group has also demonstrated a role for both adenosine and PKC in triggering and signaling associated with preconditioning in this model (147). More recently, work by Arstall et al. (15) provided direct evidence that in addition to classic preconditioning, human ventricular myocytes, in vitro, also exhibited delayed cardioprotection 24 h after a short period of simulated ischemia. Interestingly, it has also been shown that preconditioning reduced cell injury in human myocytes but not in human endothelial cells (314), although others, using human coronary arteriolar endothelial cells triggered by hypoxic preconditioning, demonstrated an upregulation of prosurvival kinases (414).

2. Human muscle preparations

Yellon’s group (371) were the first to investigate the phenomenon of preconditioning using human atrial muscle obtained from patients undergoing coronary artery bypass surgery. The atrial muscle, suspended in an organ bath, was subjected to sustained periods of simulated ischemia followed by reoxygenation before a sustained lethal hypoxic insult followed by reoxygenation. They were able to precondition human muscle using posts ischemic recovery of mechanical function as the end point (371). They also demonstrated that adenosine A1 and A3 as well as δ-opioid receptor activation all could trigger this protection (37, 57) and that both PKC and the K_ATP channel appear to be involved in mediating the protection in human muscle (56, 58, 59, 317). These results have been confirmed by Cleveland et al. (70). Furthermore, as with
animal studies, early evidence suggests that mitoK$_{\text{ATP}}$ channels may also be involved in mediating ischemic preconditioning in the human muscle. In this respect, Yellon and colleagues (37) have shown that selective blockade of mitoK$_{\text{ATP}}$ channels with 5-HD attenuates the protection by ischemic preconditioning in human atrial trabeculae. The role of K$_{\text{ATP}}$ channels in this in vitro model was also demonstrated in a work by Cleveland et al. (69) who showed that atrial trabeculae obtained from diabetic patients on oral hypoglycemic sulfonylureas, which block K$_{\text{ATP}}$ channels, could not be protected by ischemic preconditioning. As mentioned above, other trig-
derivatives, however, is that this protection appears to be associated with opening of the mitoK$_{\text{ATP}}$ (37). Other studies, using sections taken from human right atrial muscle in which leakage of creatine kinase was used as an index of injury, have demonstrated that it is possible to precondition human muscle by both simulated ischemia as well as pharmacological means and confirm the importance of the mitoK$_{\text{ATP}}$ channel in the protection observed (118). In addition, with the use of the same model, both an early and a delayed form of preconditioning have also been identified (119).

B. In Vivo Studies

1. Exercise-induced preconditioning

It is known that some patients are able to exercise to the point that they develop angina, rest, and then continue exercising with minimal or no further development of symptoms. This phenomenon, termed warm-up or first-effort angina, was first described over 50 years ago and for many years was thought to be mediated by coronary vasodilatation and recruitment of collateral vessels resulting in improved blood supply to the ischemic myocardium during the second period of exertion (212). Some investigations, however, suggest that other mechanisms might be involved in warm-up angina. Studies examining hemo-
dynamic and metabolic characteristics during consecutive exercise testing (252), or consecutive angina resulting from pacing-induced tachycardia (382), have reported a reduction in the severity of angina and the degree of S-T segment depression during the second period of myocardial ischemia. These favorable changes were not accompanied by recruitment of collateral vessels as evidenced by similar coronary and great cardiac vein blood flows measurements. However, Okazaki et al. (252) demonstrated reduced myocardial oxygen consumption during the second period of ischemia. Similarly, Tzivoni and Maybaum (357) have demonstrated a reduction in electrocardiographic evidence of silent ischemia during successive periods of exercise. A more recent study suggests that the degree of myocardial stunning following exercise-induced myocardial ischemia may also be attenuated if the patient had performed a preceding period of exercise 30 min earlier (286). Studies investigating the tempo-
ral profile of warm-up angina have demonstrated that the duration of this phenomenon is 1–2 h after the first period of exercise, a time course that closely parallels that of classic ischemic preconditioning (320, 345).

These above findings suggest that the warm-up phe-
nomenon is at least partly due to metabolic adaptation of myocardium which induces tolerance to subsequent ischemia, a process that closely resembles ischemic preconditioning. However, studies that have examined the cellular mechanisms mediating warm-up angina do not fully support this hypothesis. For instance, inhibition of aden-
osine receptors before exercise fails to abolish the warm-up phenomenon (168). Furthermore, investigation into the role of K$_{\text{ATP}}$ channels in mediating this form of myocardial adaptation has provided conflicting results (78, 259, 350). It is therefore not clear at this point whether the adaptation observed during repeated exercise is a true representation of the preconditioning phe-
nomenon, or if other mechanisms are involved. In a recent editorial Tomai (343) suggests that preconditioning might play a role in the warm-up phenomenon but that it may be mechanistically distinct from preconditioning caused by a sudden interruption of oxygen supply.

2. Preinfarction angina

Many patients experience brief episodes of ischemia before an acute MI. It is theoretically possible that this preinfract angina has the potential to precondition the myocardium, thereby reducing infarct size and improving survival. However, this would be the case only if the infarct-related artery is reperfused in a timely fashion, because experimental evidence suggests that ischemic preconditioning delays necrosis and therefore has no effect on infarction in the territory of a completely occluded artery in which no reperfusion occurs. It is not surprising then that studies which evaluated the effects of preinfract angina before widespread use of thrombolysis did not consistently show a beneficial effect in terms of mortality and left ventricular function (32, 79, 83).

A number of more recent studies evaluated the outcome of patients suffering an acute MI in relation to the presence of preinfarction angina. For example, Kloner et al. (179), in a retrospective analysis of the TIMI-4 trial, showed that the presence of preinfract angina was associated with smaller infarct size based on peak and total creatine kinase release, improved left ventricular function with reduced incidence of congestive heart failure and shock, and reduced mortality. Similar findings have been reported by other groups (11, 154, 248, 255). Moreover,
patients who experience angina before acute MI seem to have reduced occurrence of life-threatening ventricular arrhythmias associated with reperfusion (11, 332) and a lower in-hospital, 1- and 5-year cardiac mortality rate (153, 178, 179).

Whether the protection conferred to these patients as a result of their preceding ischemic symptoms represents a form of myocardial adaptation similar to ischemic preconditioning remains a subject of debate (88). Preconditioning, by virtue of delaying myocardial necrosis and improving postsischemic functional recovery, as seen in laboratory animals, may contribute to the improved outcome in patients with preinfarct angina. Interestingly, recent evidence suggests that the time interval between the last episode of angina and the index MI is very important. Reports from the TIMI-9B investigators (178) and studies by Ishihara et al. (153) and Yamagishi et al. (389) indicate that prodromal angina is only protective if it occurs within 24–72 h of MI, a time course that closely resembles that of the delayed phase of myocardial protection following ischemic preconditioning in animal models.

However, in addition to the possible protection conferred by ischemic preconditioning, infarct size, and the degree of preservation of postsischemic left ventricular function are determined by a number of other factors including the extent of collateral circulation to the ischemic myocardium, time from onset of infarction to reperfusion of the infarct-related artery, and residual coronary stenosis after reperfusion. Studies that have analyzed the degree of collateralization to the ischemic zone after an infarction have found no increase in angiographically visible collateral vessels in patients with preinfarct angina (179, 255). It must be noted, however, that coronary angiography at 90 min after thrombolysis is unlikely to provide information about the degree of collateral recruitment to the ischemic zone during coronary occlusion, and it is therefore difficult to rule out a contribution by collateral circulation in this setting. Interestingly, in a recent study (389), resting myocardial-dual isotope SPECT using $^{125}$I-15-(p-iodophenyl)-3-(R,S)-methylpentadecanoic acid ($^{125}$I-BMIPP) and thallium ($^{203}$TI) in the subacute phase of MI was employed to assess the area at risk and necrotic myocardium, respectively. Yamagishi et al. (389) found that patients with preinfarct angina had significantly smaller infarcts compared with those with no preceding symptoms, in the face of no significant difference in areas of myocardium at risk, suggesting that the presence of preinfarction angina did not predict improved collateral recruitment to the ischemic zone.

Another equally attractive hypothesis, although not mutually exclusive from the mechanisms underlying ischemic preconditioning, is facilitation of more rapid reperfusion of the infarct-related artery following thrombolysis in patients with preinfarct angina (9). This hypothesis is based on the known inhibitory effects of adenosine, released during the brief periods of preinfarct ischemia, on platelet aggregation following activation of A$_2$ receptors on platelet membranes, which has been suggested to modify thrombus formation and thereby promote earlier reperfusion after thrombolysis (8). In this regard, Przyklenk and colleagues (136, 280) have recently demonstrated that in anesthetized open-chest dogs, brief periods of ischemia before a long ischemic insult attenuates platelet-mediated thrombosis and improves vessel patency and that this effect was abolished by inhibition of adenosine receptors.

It must be stated that not all preinfarct angina studies have yielded positive results. Zahn et al. (411) in a recent report demonstrated that preinfarct angina had no benefit when the infarct area was perfused by PTCA rather than thrombolysis, which may well support the findings of Andreotti et al. (9).

In addition to the above, there is also debate as well as controversy as to whether preconditioning occurs in the elderly patient or indeed if it can be of any benefit (1–3, 160, 176). Although most of the preclinical experimental data would suggest that preconditioning the aged animal may present a problem (106, 302) in the clinical setting, this is not as clear.

3. Angioplasty studies

Coronary angioplasty (PTCA) provides a unique opportunity to study the response of the human myocardium to brief periods of controlled ischemia and reperfusion. The procedure usually involves repeated intracoronary balloon inflations with intervening periods of perfusion, and in theory the first period of ischemia may enhance the myocardial tolerance to subsequent balloon inflations via classic ischemic preconditioning. Several recent studies have addressed this issue using various indices of myocardial ischemia including clinical, electrocardiographic, metabolic, and hemodynamic measurements. Most of these studies, but not all (99), have shown that if the duration of the first balloon inflation is longer than a “threshold” of ~60–90 s, all indicators of myocardial ischemia, including chest pain severity, abnormalities of left ventricular regional wall motion, S-T segment elevation, Q-T dispersion, ventricular ectopic activity, lactate production, and release of myocardial markers such as CKMB are attenuated during subsequent balloon inflations, providing evidence for myocardial adaptation induced by the first period of ischemia (4, 50, 92, 103, 253). As with many studies of ischemic preconditioning in humans, a major confounding factor during successive balloon inflations in PTCA studies is the acute recruitment of collateral vessels. However, studies that have controlled for this effect by angiographic grading of the collateral vessels (80), measurement of cardiac vein flow (92),
changes in blood flow velocity in the contralateral coronary artery (348), and more accurately, by assessment of intracoronary pressure derived-collateral flow index during successive balloon inflations (40), have shown that although collateral recruitment occurs in some patients, it cannot fully explain the myocardial adaptation observed during repeated balloon inflations. Furthermore, some would argue that collateral flow recruitment in the setting of coronary angioplasty does not make a major contribution to myocardial protection in this setting (218).

Investigation into the mechanisms underlying this rapid protection of the myocardium during PTCA has provided further support for a preconditioning-like effect. Tomai et al. (346) reported that blockade of K\textsubscript{ATP} channels with oral glibenclamide before angioplasty abolishes the reduction in ischemic indices observed during subsequent balloon inflations, implying a role for these channels in mediating this form of adaptation (see Fig. 6). This finding is supported by the observation that opening of these channels with nicorandil reduces the electrocardiographic indices of ischemia during coronary angioplasty (288). Furthermore, an important role has been demonstrated for adenosine in mediating myocardial adaptation during coronary angioplasty (195). Inhibition of adenosine receptors by bamiphylline (347) or aminophylline (66) abolishes myocardial adaptation during the second balloon inflation. Conversely, intracoronary infusion of adenosine before PTCA, independent of its vasodilatory effect, attenuates ischemic indices during the first balloon inflation (195). Two other reports have suggested a role for both opioid (349) and bradykinin (197) receptors in mediating myocardial adaptation during PTCA. These studies provide further evidence that myocardial tolerance to further ischemic episodes can be induced by...
preceding brief periods of ischemia and that this tolerance may be mediated by the same mechanisms as those involved in ischemic preconditioning in animal models. With respect to the SWOP, very little information is available as to whether pharmacological protection can be seen in humans. Leesar et al. (196) were the first to report that a delayed protective effect could be seen in patients undergoing angioplasty using nitroglycerin and suggest that prophylactic administration could be a novel approach to protection of the ischemic myocardium in such patients. However, the potential development of tolerance seen with this type of agent may preclude its prophylactic use (139).

However, recent experimental evidence has provided grounds for caution when interpreting the results of these PTCA studies, which have mostly employed S-T segment elevation as an end point reflecting the degree of myocardial ischemia, and its attenuation during successive balloon inflations as an indicator of enhanced myocardial resistance to ischemia. Although this assumption was supported by earlier experimental studies of repeated coronary artery occlusion in collateral-deficient pig and rabbit hearts (74, 308), a study by Downey’s group (41) clearly indicates a dissociation between S-T segment changes on the electrocardiogram and myocardial protection in terms of infarct limitation. Their finding, that the changes in S-T segment voltage during coronary artery occlusion may merely represent an epiphenomenon distinct from the cardioprotective effect of ischemic preconditioning, is particularly pertinent when evaluating or designing mechanistic studies using pharmacological agents to mimic or abolish the cellular signaling mechanisms of ischemic preconditioning. It is imperative that the influence of these pharmacological tools on the sarcolemmal K$_{ATP}$ channels, thought to modulate electrocardiogram voltages, is clearly distinguished from their effect on the mitoK$_{ATP}$ channels, which have been proposed as a mediator of cardioprotection (297).

As with preconditioning in elderly patients in the setting of warm-up angina, Lee et al. (194) noted that in the setting of angioplasty there also appeared to be impaired preconditioning responses in elderly compared with adult patients that was related to ATP-sensitive potassium channels.

4. Surgical studies

Possibly the most direct evidence for preconditioning in humans comes from studies that have examined whether a specified preconditioning protocol can protect the human myocardium from the period of global ischemia induced by aortic cross-clamping during coronary artery bypass grafting. In this respect, Yellon et al. (403) were the first to report a prospective study examining the effects of a preconditioning protocol of two cycles of 3 min of global ischemia (induced by intermittent cross-clamping the aorta and pacing the heart at 90 beats/min) followed by 2 min of reperfusion before a 10-min period of global ischemia and ventricular fibrillation. Changes in ATP content from needle biopsies of left ventricular muscle were used as the end point in this study. It was found that patients subjected to this preconditioning protocol had better preservation of ATP levels during the subsequent global ischemic period. These findings were almost identical to those observed in canine hearts by Murry et al. (241). The preconditioned dogs showing this relative preservation of ATP during the early stages of prolonged ischemia sustained significantly smaller infarcts at the end of 40-min ischemia. Myocardial necrosis was not estimated in that original human study; however, in a more recent study (157), serum levels of troponin T were used as an indicator of myocardial cell necrosis in the same model (see Fig. 7). With the use of this end point, it was shown that patients subjected to the preconditioning protocol suffered significantly less myocardial necrosis during the 10-min period of global ischemia. This study has recently been repeated and similar results obtained with ischemic preconditioning (338). In an earlier study, cardioplegic arrest was used as opposed to cross-clamp fibrillation. A preconditioning protocol of 1 min of aortic cross-clamping followed by 5 min of reperfusion immediately before the cardioplegic arrest resulted in a significant improvement in postoperative cardiac index and reduced requirement for inotropic support compared with the nonpreconditioned group (150). In addition to the above, there have been some studies that have compared the use of ischemic preconditioning with that of pharmacological preconditioning with mixed results. In this regard, using troponin I release as an index of injury, it has been shown that preconditioning with a 5-min infusion of adenosine, followed by 10-min washout, failed to demonstrate any protection compared with control (34). More recently, Yellon’s group (338) has shown that an adenosine A$_1$ receptor agonist, GR79123x, although statistically unable to demonstrate protection compared with preconditioning with ischemia, did appear to offer some benefit. As discussed in section AC, it is known that to precondition the myocardium a certain threshold of stimulation has to be reached to elicit the preconditioning response (122, 236). In addition, as discussed above, the duration of ischemia required to elicit preconditioning is known to vary between species (192). Thus it could be argued that the dose of both the adenosine (34) and GR79236x (338) used may have been insufficient to reach the threshold to trigger the myocardial preconditioning signaling pathway in humans. Another possible explanation is that stimulation of the adenosine A$_1$ receptor alone is inadequate to precondition the human heart in this setting. Evidence exists for a role for both the adenosine
A1 and A3 receptors in protection of both isolated rabbit as well as isolated human atrial muscle against simulated ischemia. As such, stimulation of both A1 and A3 adenosine receptors may be required to precondition the human heart in vivo.

In addition to the above, it could also be argued that many drugs used during cardiac surgery are noted to have preconditioning properties in the laboratory setting. In this regard, isoflurane has been shown to have beneficial effects during coronary artery bypass surgery (using troponin I release as an index of injury) (351). Isoflurane itself has also been used to directly precondition the myocardium when given as a preconditioning stimulus (34, 77). In addition, it is well known that opioids can precondition both animal and human muscle (37, 202).

Studies that have used other cardioprotective strategies during the prolonged period of ischemia, such as hypothermia or cardioplegia, have not consistently demonstrated additional protection by ischemic preconditioning. For instance, Perrault and colleagues (269), using a similar preconditioning protocol of one 3-min episode of aortic cross-clamping before the onset of cardioplegic arrest, failed to show any beneficial effects compared with the control group; in fact, the preconditioned group of patients had more creatine kinase release compared with case-matched controls. Similarly, negative results have been reported by another group using normothermic retrograde blood cardioplegia with or without preceding ischemic preconditioning (167). These divergent results have led to the hypothesis that in the setting of coronary artery bypass surgery, the additional protection conferred by ischemic preconditioning may only be demonstrable where a potential for suboptimal myocardial protection increases the risk of perioperative infarction (268).

C. Therapeutic Implications

It can be deduced from the evidence outlined above that the human myocardium is amenable to preconditioning and also that preconditioning occurs as a natural feature of some ischemic syndromes. Two important questions arise regarding the applicability of ischemic preconditioning that need careful consideration. First, are there areas in clinical medicine in which therapeutic preconditioning may be utilized to benefit patients with ischemic heart disease? Second, can the naturally occurring preconditioning that follows ischemic syndromes such as angina be exploited, and does treating the symptoms of angina abolish any possible cardioprotective effects?

Early revascularization strategies remain the most effective means of limiting ischemic injury. The time in-
terval between the onset of symptoms and initiation of revascularization is however crucial, and the benefits of treatment diminish as this interval increases (341). Preconditioning, by virtue of delaying myocardial necrosis, prolongs the time window during which revascularization therapies can be administered. However, the use of brief antecedent ischemia as a means of prophylactic induction of this protection is not desirable or feasible in most circumstances. On the other hand, the use of pharmacological agents capable of mimicking the protective effects of preconditioning, in lieu of brief ischemia, may provide a more benign approach for eliciting cardioprotection. Potential candidates currently in clinical use include adenosine or its analogs and K<sub>ATP</sub> channel openers such as nicorandil. It is crucial to appreciate however that such strategies require pretreatment; the myocardium must be preconditioned before the onset of lethal ischemia. Of recent interest in this respect are the results from the IONA study (339). This study was the first outcome study in stable angina which reported that patients pretreated with the K<sub>ATP</sub> channel opener nicorandil had an improved outcome due a reduction in major coronary events. Although the trial design does not permit any conclusions as to the precise mechanism through which the beneficial effects were seen, the authors suggest that the most plausible explanation is that nicorandil acts as pharmacological mimic of the preconditioning phenomenon.

Whether this agent is the first clinically available preconditioning mimetic or not, it is clear that our understanding of the potential mechanisms associated with the preconditioning phenomenon has allowed agents such as nicorandil, a known mitoK<sub>ATP</sub> channel opener (296), to be examined in the clinical setting. It is also possible that other drugs that potentiate preconditioning triggers such as ACE inhibitors could also have influenced the clinical outcome from some of the trials such as HOPE (410).

The acute coronary syndromes (ACS) comprise a spectrum of pathophysiological conditions spanning unstable angina, non-S-T elevation MI and acute S-T elevation MI. In patients with acute MI with persistent S-T elevation, early reperfusion to reestablish epicardial blood flow is well established as the standard of care, be it with early fibrinolytic therapy, or where the facilities and expertise are available, to undertake primary angioplasty (353). As far as pharmacological preconditioning strategies are concerned, these patients are unlikely to benefit from such treatment, and their management should focus on early restoration of coronary artery patency and potential strategies to minimize reperfusion injury (405). On the other hand, non-S-T elevation ACS, including unstable angina and non-Q-wave MI, mark the transition from stable coronary artery disease to an unstable state and constitute the leading cause of hospital admission in patients with coronary artery disease. This group of patients is at a high risk of progression to acute coronary occlusion, and >10% die or suffer a MI (or reinfarction) within 6 mo with about one-half of these events occurring during the acute early phase (402). Thus this group of patients may be amenable to pharmacological intervention with agents that mimic the preconditioning phenomenon to provide potential “insurance” should that patient proceed to infarction or to buy time before instituting some form of reperfusion strategy. In this regard, Patel et al. (264) have shown that opening of K<sub>ATP</sub> channels with nicorandil, in addition to standard aggressive medical therapy for unstable angina, results in a significant reduction in the incidence of myocardial ischemic episodes and tachyarrhythmias. This may purely represent an anti-ischemic effect due to the vasodilatory properties of nicorandil. However, because the patients in this study were already on maximal antiangiinal therapy, and in particular a significant proportion were treated with intravenous or oral nitrates, it is possible that the protection observed in the nicorandil group, be it only using soft end points of myocardial injury, may at least partially be due to a preconditioning-like effect (89).

Second, even when prior treatment with the pharmacological preconditioning agent is feasible, the duration of the protection afforded is limited. The temporal profile of the protective effects of preconditioning in humans is unknown, but according to experimental evidence in laboratory animals, it is unlikely to exceed 48–72 h (26, 334). Therefore, unless the onset of an ischemic event can be predicted with accuracy, repeated dosing with the potential preconditioning drug will be necessary in these high-risk patients to maintain the preconditioned state. Early experimental evidence suggested that the protective effects of “classic” ischemic preconditioning is lost after prolonged periods of repetitive ischemia (73), or chronic pharmacological preconditioning with selective adenosine A<sub>1</sub> agonists (355). However, encouraging evidence indicates that tachyphylaxis could be overcome by exploiting the prolonged time course of the SWOP. Intermittent treatment of conscious rabbits with an optimal dosing regimen of pharmacological preconditioning with selective adenosine A<sub>1</sub> receptor agonists maintains the animals in a preconditioned state over a period of several days and results in a significant reduction in infarct size (84).

Preconditioning strategies might also be applied before planned procedures that involve a potentially injurious ischemic insult such as coronary artery bypass surgery or angioplasty. Highly effective methods of myocardial protection during coronary artery surgery have been developed including chemical cardioplegia, hypothermia, and cross-clamp ventricular fibrillation. However, with increasing number of operations on older and higher risk patients, there is always a need for improved protection. Therapies that stimulate preconditioning mechanisms, administered before such operations, have the potential to...
provide this increased protection. For instance, adenosine, an important mediator of the preconditioning phenomenon in human atrial trabeculae (371) and during coronary angioplasty (195), has been shown to result in improved postoperative left ventricular function when administered intravenously before cardiopulmonary bypass (193). Similarly, although routine PTCA carries a small risk (<5%) of complete coronary occlusion and MI, high-risk patients undergoing PTCA might benefit from pretreatment with agents that mimic preconditioning or augment the protection afforded by the first balloon inflation. In this respect, a recent study by Sakai et al. (289) has demonstrated that an intracoronary infusion with the nicorandil prior to PTCA resulted in enhanced tolerance to subsequent prolonged balloon inflations. The possibility that organ preservation before transplantation might be amenable to the same improved protection, as suggested by some experimental evidence (164a, 166, 190), is also of significant interest. This might allow an extension of the “cold ischemic time” between harvesting and implantation, facilitating optimal matching of recipient to donor, as well as affording a potential improvement in early myocardial function.

In response to the second question of whether angina induces preconditioning and if treating the symptoms of angina abolish this protection, the evidence must be viewed with caution. It must be emphasized that extension of the evidence reviewed above in favor of preconditioning to routine clinical practice is speculative at this stage. However, considering the current evidence for the mechanisms underlying myocardial adaptation, we can question the choice of drug treatment for patients with angina. For example, the probable involvement of $K_{ATP}$ channels as protein mediating the cardioprotective effects of preconditioning favors the use of the $K_{ATP}$ channel openers such as nicorandil. Conversely, the increased mortality from ischemic heart disease observed in diabetic patients on certain oral hypoglycemic therapy begs a radical review of drug treatment of diabetic patients with angina (104), since sulfonylureas, the most widely used oral hypoglycemic agents, block $K_{ATP}$ channels and the possible cardioprotective effects of ischemic preconditioning. In this regard, it has recently been shown that not all sulfonylureas appear to behave in the same way with respect to preconditioning. Using an isolated perfused rat heart model, Yellon and colleagues (234) were able to demonstrate that glibenclamide blocked the protective effect of preconditioning; this effect was directly attributable to the mito$K_{ATP}$ channel, whereas a newer sulfonylurea, glimeperide, with less cardiovascular specificity, was unable to block the protection or effect the mito$K_{ATP}$ channel. Such studies may have important implications for the treatment of type II diabetic patients with ongoing ischemic heart disease. Similarly, the demonstration that antagonism of adenosine receptors prevents ischemic preconditioning during coronary angioplasty (66, 348) and in human atrial trabeculae (371) questions the use of agents such as methylxanthines in high-risk patients such as those with unstable angina or those undergoing revascularization procedures.

D. Summary

A wealth of evidence supports the concept that ischemic preconditioning profoundly and consistently limits infarct size in the experimental laboratory. Several lines of evidence obtained from human myocardial tissue, from the catheterization laboratory and the operating theater, and from analyses of large clinical trials suggest that the human myocardium may behave in a similar way. There are several classes of pharmacological agents that may be able to mimic the protection conferred by ischemic preconditioning and provide some basis for optimism that a beneficial and clinically detectable improvement in myocardial protection may be possible. In the short term, further studies in routine (low-risk) patients must be performed to establish the safety of these agents using multiple end points to detect small differences in myocardial viability and extent of microinfarction. In the longer term however, large-scale studies involving high-risk patients are warranted to investigate the potential cardioprotective effects of these agents with comparisons against preexisting myocardial protective strategies. With a more complete understanding of the mechanisms underlying myocardial adaptation, we can look forward to development of new therapeutic agents with novel mechanisms of action to supplement the current treatment options for patients with ischemic heart disease.

VI. CONCLUSION AND PERSPECTIVES

In conclusion, the phenomenon of ischemic preconditioning has shown us that cardioprotection is indeed possible. The exquisite adaptive behavior of the cell in protecting itself from severe ischemic stress is truly remarkable. Over the last two decades there has been a concerted effort of trying to understand the mechanism of this adaptive phenomenon. Clearly significant inroads have and are continuing to be made into the mechanism of this phenomenon. This review has tried to put in context the enormous research effort that has taken place over the last two decades; however, we must not be complacent and must continue until a complete understanding of the preconditioning phenomenon has been achieved.

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MYOCARDIAL PRECONDITIONING

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