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REVIEW

Role of bradykinin in preconditioning and protection of the ischaemic myocardium

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Abbreviations: ACE, angiotensin converting enzyme; ATP, adenosine triphosphate; MPG, mercaptopropionylglycine; NEP, neutral endopeptidase; NO, nitric oxide; NOS, nitric oxide synthase; PKC, protein kinase C; 8-SPT, 8-(p-sulphophenyl)theophylline

Introduction

Since the first description of bradykinin more than 50 years ago (Rocha e Silva *et al*., 1949), actions of the peptide in a variety of physiological and pathological responses have been extensively researched (reviewed in Bhoola *et al*., 1992; Wirth *et al*., 1997; Calixto *et al*., 2000). In the cardiovascular system, the classical action of bradykinin is vasodilatation, mediated in several vascular beds by the release of nitric oxide (NO) and prostacyclin (Hatta *et al*., 1997; Wirth *et al*., 1997). In the heart, exogenously-administered bradykinin is a potent coronary artery vasodilator substance, although the contribution of endogenous bradykinin to the regulation of coronary vascular tone is unclear. Several actions of bradykinin in the heart are of particular interest as they are independent of the vasodilator actions of the peptide. Such actions include the modulation of cell growth and division in the heart and the modulation of myocardial responses to ischaemia-reperfusion. The ability of bradykinin to act as an endogenous cytoprotective mediator in the ischaemic myocardium has received a great deal of attention in recent years. Much of this research on bradykinin has been fuelled by a growing appreciation of ischaemic preconditioning, an adaptive mechanism in which bradykinin plays an important role. This review focuses on the cytoprotective actions of bradykinin in the ischaemic and reperfused myocardium, discusses its role in the ischaemic preconditioning response, and examines the potential for manipulating endogenous bradykinin for therapeutic benefit in myocardial ischaemia.

Myocardial ischaemia-reperfusion injury

Acute thrombotic occlusion of a major coronary artery, leading to myocardial ischaemia, is a leading cause of death and morbidity in the industrialized and developing countries. Ischaemia rapidly produces profound metabolic, functional and morphological changes within myocardium, the severity of which are ultimately determined by the duration of impaired oxygenation and substrate delivery (Ganz & Braunwald, 1997). The principal metabolic changes centre around the failure of adequate adenosine triphosphate (ATP) generation by oxidative phosphorylation and the accumulation of byproducts of anaerobic glycolysis, particularly H^+. The functional consequences of ATP depletion are rapidly manifested as a decrease in contractility and disturbances of a host of homoeostatic processes, including the activities of ion channels and exchangers, cell volume regulation and enzyme reactions. The electrical properties of ischaemic myocardium may be altered to the point where arrhythmogenic mechanisms can promote life theatening tachyarrhythmias. Ultrastructural changes may be detectable within several minutes of the onset of ischaemia. These alterations may be considered reversible if reperfusion of the tissue can be effected promptly. However, ischaemia lasting more than 20–30 min will result in irreversible cell injury (Schaper *et al*., 1992). Without reperfusion to salvage ischaemic myocardium, the most extreme manifestation of irreversible injury is tissue necrosis (myocardial infarction). Prompt reperfusion of the occluded vessel is required to save ischaemic myocardium from sustaining irreversible injury but, paradoxically, reperfusion may be associated with further cellular stress resulting in 'reperfusion injury'. The development of therapeutic strategies that can attenuate ischaemia-reperfusion injury has been a keen area of research for more than 30 years.

Ischaemic preconditioning of myocardium

Brief antecedent episodes of ischaemia enhance tissue tolerance to a subsequent longer episode of ischaemia. This phenomenon was formally described by Murry *et al*. (1986) who coined the term 'preconditioning with ischaemia'. They demonstrated in canine myocardium that four short non-injurious coronary artery occlusions, before a subsequent 40 min coronary occlusion, reduced the development of necrosis during the 40 min occlusion by almost 75% compared to the necrosis in non-preconditioned hearts. This powerful protective effect of

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antecedant ischaemia was not explained by changes in coronary collateral blood flow, suggesting a fundamental cellular alteration in the response to ischaemia. The protection conferred by preconditioning with ischaemia has been subsequently confirmed in many studies and has excited a huge effort to determine the underlying molecular mechanisms of protection (Cohen *et al*., 2000).

Preconditioning protocols vary somewhat from one laboratory to another and many endpoints of ischaemic injury have been adopted to assess the extent of protection conferred by preconditioning, including development of necrosis, severity of arrhythmias, post-ischaemic recovery of contractile function and cardiac enzyme release. Striking features of preconditioning are the temporal aspects of the protection. The protection conferred by preconditioning is not absolute in as much as preconditioning in canine myocardium limits infarction produced by a 40 min coronary occlusion but does not protect against a 180 min occlusion (Murry *et al*., 1986). Protection is lost if the reperfusion period between the brief preconditioning ischaemia and the long ischaemic period is extended beyond 2 or 3 h (van Winkle *et al*., 1991; Kuzuya *et al*., 1993). However, a further period of protection may be detected many hours later suggesting a biphasic pattern of protection. The early phase of protection, of rapid onset and short duration is the classic preconditioning effect, originally described by Murry *et al*. (1986), while the delayed phase occurring many hours later and lasting much longer, has been termed 'second window of protection', 'delayed preconditioning' or 'late preconditioning' (Bolli, 2000; Baxter & Ferdinandy, 2001).

Paracrine and autocrine triggers of classical preconditioning

Extensive research has revealed that several endogenous mediators of myocyte, endothelial and neural origin, are generated during the brief preconditioning period, and these act as co-activators (or 'triggers') of a signal transduction cascade that rapidly results in the acquisition of tolerance to further ischaemia (see Figure 1). Detailed discussion of the involvement of multiple kinase families is beyond the scope of this review and has been comprehensively reviewed elsewhere (Cohen *et al*., 2000; Baines *et al*., 2001). The earliest mediator to be examined as an activator of preconditioning was adenosine. In several species, adenosine, released from myocytes as a consequence of ATP breakdown during preconditioning, acts on adenosine A_1 receptors and possibly A_3 receptors, initiating a signaling cascade. Early experimental evidence to support the involvement of adenosine came from Liu *et al*. (1991) who showed that adenosine receptor blockade with 8-p-(sulphophenyl)-theophylline (8-SPT) during preconditioning could abolish the infarct-limiting effect. Conversely, transient adenosine A_1 receptor activation, but not A_2 receptor activation, with selective agonists mimicked the preconditioning effect of brief coronary occlusion in the rabbit (Thornton *et al*., 1992; Tsuchida *et al*., 1993). A role for adenosine A_3 receptor activation has been proposed by some workers (Armstrong & Ganote, 1994; Liu *et al*., 1994) but is not clearly resolved (Guo *et al*., 2001; Kilpatrick *et al*., 2001). It has since become clear that several other endogenously liberated autocrine/paracrine mediators contribute critically to initiating the process of cellular adaptation. All of these mediators are released or rapidly generated during relatively brief periods of myocardial ischaemia and they include catecholamines (Bankwala *et al*., 1994), opioid peptides (Schultz & Gross, 2001), reactive oxygen species (Baines *et al*., 1997) and bradykinin, which is the focus of this article.

Formation and catalytic degradation of bradykinin

Bradykinin is one of several oligopeptides called kinins. The most important physiologically active kinins are kallidin (Lys-bradykinin), bradykinin, and des-Arg^9-bradykinin. The interested reader is referred to a recent review by Blais *et al*. (2000) for a detailed account of kinin synthesis. Kallidin and bradykinin are synthesized by kallikreins acting on kininogen precursor molecules (summarized in Figure 2a). Precursors of kallikreins are found in plasma (pre-kallikreins) and in tissues (pro-kallikreins) and are activated by a variety of chemical and biological stimuli, including activated factor XII (Hageman factor). Circulating kininogens are synthesized primarily in liver and include a high molecular weight kininogen (88–115 kDa according to species) and a low molecular weight kininogen (50–68 kDa).

Vascular endothelial cells are the primary source of bradykinin in the heart (Linz *et al*., 1996; Wirth *et al*., 1997). Enzymatic cleavage of pre-kallikrein generates kallikrein at the endothelial cell surface. Circulating kininogen is then cleaved by kallikrein to generate the kinin at the endothelial cell surface. The mechanisms by which pre-kallikrein is activated may be factor XII-dependent or -independent. In the absence of endothelial cell injury and hence contact binding of factor XII, the mechanism of kininogen attraction may involve a cell surface receptor complex. However, this mechanism is presently not well understood. It has been proposed that isolated cardiac myocytes can synthesize kinins (Matoba *et al*., 1999) but this possibility remains to be investigated more fully. A number of studies have provided evidence that even during brief preconditioning periods of ischaemia tissue and plasma bradykinin levels increase markedly (Linz *et al*., 1996; Schulz *et al*., 1998; Campbell, 2000; Pan *et al*., 2000). Bradykinin is generated in isolated tissues and endothelial cells in the absence of plasma (Baumgarten *et al*., 1993; Ahmad *et al*., 1996; Linz *et al*., 1996). Bradykinin released during ischaemia has been shown to primarily originate from endothelial cells (Linz *et al*., 1996; Wirth *et al*., 1997) but the precise molecular pathological mechanism leading to bradykinin generation during brief ischaemia is not understood.

Once released, bradykinin is rapidly degraded into inactive metabolites (Bhoola *et al*., 1992) (Figure 2b). Enzymes that degrade bradykinin are generically referred to as 'kininases' or 'kinin peptidases'. The most important of these metalloproteases are angiotensin converting enzyme (ACE; syn. kininase II; EC 3.4.15.1), neutral endopeptidase (NEP; syn. NEP 24.11; enkephalinase; EC 3.4.24.11), kininase I (syn. carboxypeptidase N; EC 3.4.17.3), carboxypeptidase M (syn. membrane-bound kininase I), and aminopeptidase P (syn. prolyl-aminopeptidase; EC 3.4.11.9). Other enzymes include endopeptidase (EC 3.4.24.15), endothelin converting enzyme (ECE) and prolyl

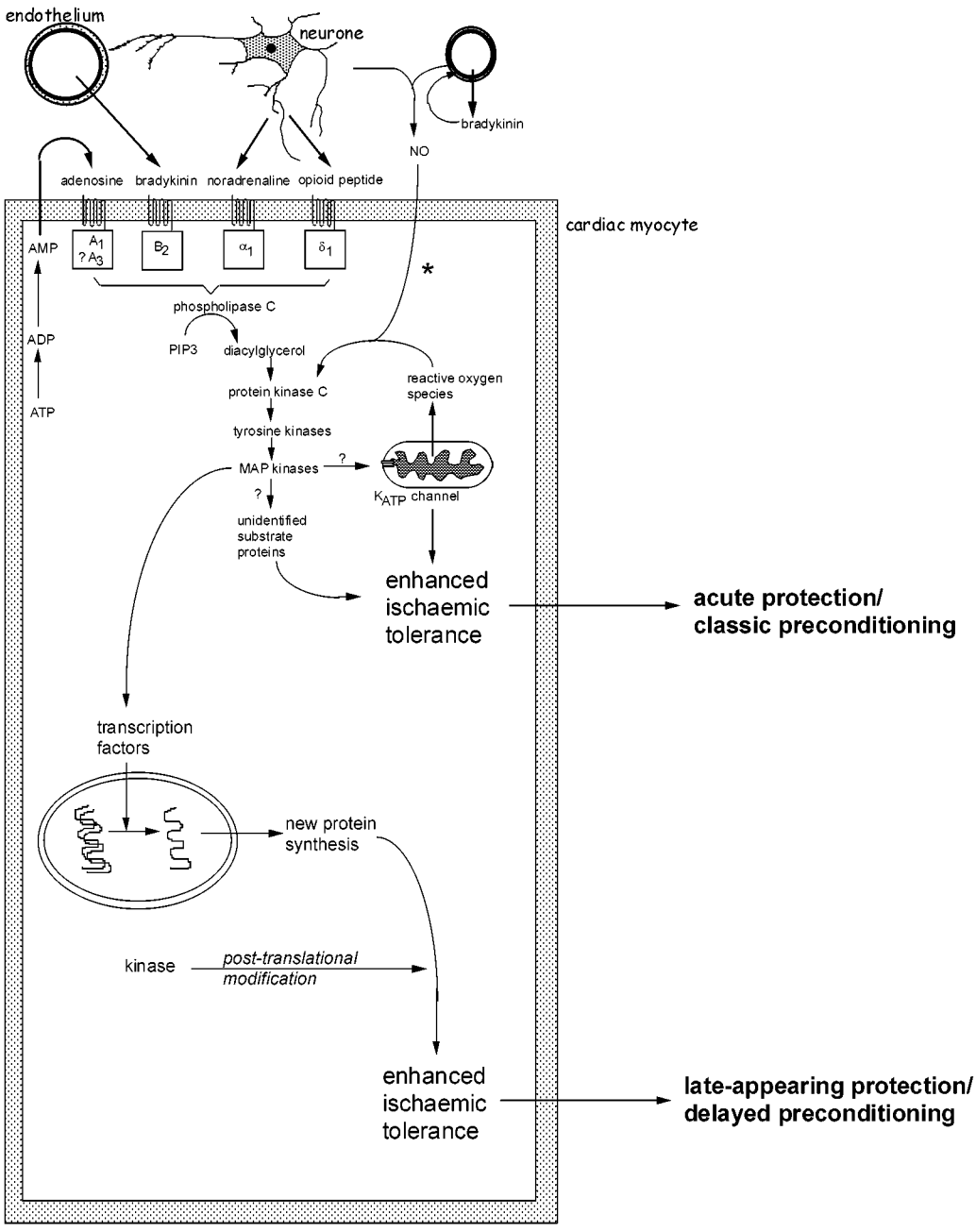

Figure 1 Schematic representation of the major identified pathways of early and delayed forms of preconditioning. Several autocrine/paracrine mediators released during the period of preconditioning ischaemia act on G-protein coupled receptors and are known to participate in the infarct-limiting effect of ischaemic preconditioning. These include adenosine released during ischaemia as a result of ATP breakdown, bradykinin released from vascular endothelium and mediators of neural origin (noradrenaline and opioid peptides). Reactive oxygen species, especially superoxide anion generated as a result of mitochondrial uncoupling, may also act as upstream mediators. A complex signal cascade is activated which involves activation of protein kinase C isoenzymes, tyrosine kinases and mitogen-activated protein kinases. The phosphorylation cascade is thought to result in activation of the ATP-sensitive potassium (K_{ATP}) channel on the mitochondrial inner membrane. At present it remains unknown how opening of this channel confers protection during ischaemia. The participation of other 'cytoprotective' proteins has been proposed, including proteins that suppress or modulate apoptosis and proteins associated with cytoskeletal integrity (αB-crystallin and 27 kDa heat shock protein). *The participation of endogenous NO (of endothelial or neural origin) in initiating the classical preconditioning mechanism may be model specific. Early protection against cell death and infarction is not NO-dependent whereas preconditioning against arrhythmias does involve NO generation. For delayed preconditioning, evidence for the involvement of NO (possibly as a signalling intermediate downstream of bradykinin) is more persuasive and consistent. The distinguishing feature of delayed preconditioning is the co-ordinated regulation of a gene transcription programme as a result of upstream kinase signalling. The delayed phase of protection is dependent on *de novo* synthesis of inducible proteins. Those thought to be particularly important in the acquisition of delayed tolerance to ischaemia include iNOS, cyclo-oxygenase-2 and intracellular antioxidant enzymes such as manganese–superoxide dismutase. For more detailed discussion see Baxter & Ferdinandy (2001).

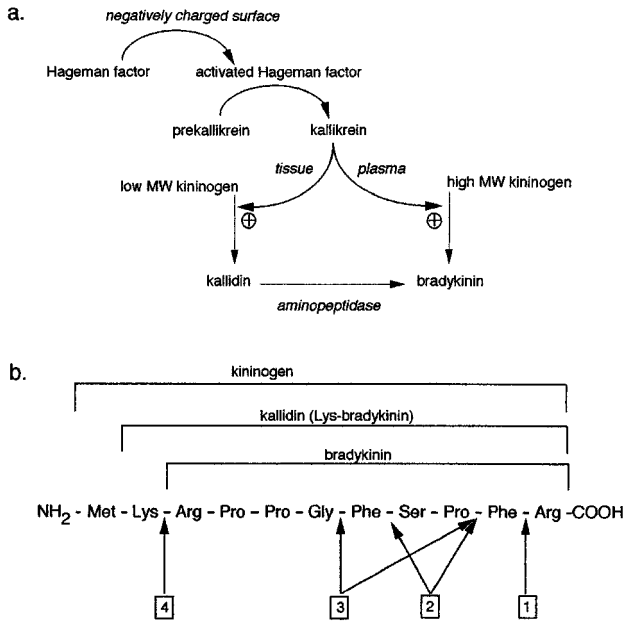

Figure 2 (a) Synthesis of bradykinin. The activated form of Hageman factor promotes the conversion of prekallikrein to kallikrein. In rat, both plasma and tissue kallikrein catalyse the formation of bradykinin. In humans, however, plasma kallikrein generates bradykinin using high molecular weight kininogens and tissue kallikrein generates kallidin using low molecular weight kininogens. (b) Basic amino acid sequence of bradykinin and precursors. Arrows designate the peptide bonds cleaved by: 1 kininase-I; 2 angiotensin converting enzyme (kininase-II); 3 neutral endopeptidase; 4 aminopeptidase.

endopeptidase but their contribution is small (Brown & Vaughan, 1998; Erdos & Skidgel, 1997; Piedimonte *et al*., 1994). ACE is regarded as the most important kininase in most species (Ahmad *et al*., 1996; Dumoulin *et al*., 1998; Ersahin & Simmons, 1997; Hornig *et al*., 1997; Kuoppala *et al*., 2000). ACE has a higher affinity for bradykinin than for angiotensin I, resulting in more favourable kinetics for bradykinin than for angiotensin I degradation (Zisman, 1998). Hence ACE may be regarded as being primarily a kininase rather than an angiotensinase (Blais *et al*., 2000).

The actions of kinins are mediated by two receptor subtypes, distinguishable on the basis of both pharmacological and molecular characterization (Hall, 1992; 1997). The bradykinin B_2 receptor usually predominates, with the bradykinin B_1 receptor only being expressed under pathological conditions (Bhoola *et al*., 1992). The B_2 receptor belongs to the family of heptahelical G-protein coupled receptors, which initiate the generation of inositol triphosphate and diacylglycerol, with subsequent activation of PKC (Derian & Moskowitz, 1986; Minshall *et al*., 1995; Morgan-Boyd *et al*., 1987). Highly specific antagonists at the B_2 receptor include the bradykinin-derivative icatibant (HOE140) and the non-peptide FR173657 (Aramori *et al*., 1997).

Endogenous bradykinin and its role in ischaemic preconditioning

Schoelkens *et al*. (1988) were the first to report the cardioprotective effects of exogenously administered bradykinin. In an isolated working rat heart preparation subjected to ischaemia followed by reperfusion, perfusion with bradykinin 10^{-10} mol l^{-1} resulted in better recovery of coronary flow and cardiac work during reperfusion, a reduction in the release of soluble markers of tissue injury, and improvement of metabolic efficiency. Following this report, intracoronary bradykinin administration, at a dose that did not induce coronary vasodilatation, was found to suppress both ischaemia- and reperfusion-induced arrhythmias in an anaesthetized canine model of epicardial coronary artery occlusion (Végh *et al*., 1991). In a porcine coronary occlusion model, infusion of bradykinin after the onset of coronary occlusion was found to attenuate plasma creatine kinase concentration. (Tio *et al*., 1991; Tobe *et al*., 1991). Subsequently, Végh *et al*. (1993) showed the anti-arrhythmic effect of bradykinin to be mediated by NO. These workers suggested that bradykinin might be a 'primary mediator' of ischaemic preconditioning.

In 1994, two reports provided evidence for a primary role of endogenous bradykinin in mediating ischaemic preconditioning. Wall *et al*. (1994) reported that icatibant abolished the protective effects of preconditioning against infarction in an anaesthetized open-chest rabbit preparation with coronary artery occlusion. They also found that protection, comparable to that induced by preconditioning, could be produced by direct infusion of exogenous bradykinin (Wall *et al*., 1994). Almost simultaneously, Végh *et al*. (1994) documented the abrogation by icatibant of the anti-arrhythmic effects of preconditioning in the canine coronary occlusion model. Goto *et al*. (1995) subsequently confirmed the finding of Wall *et al*. (1994) that icatibant blocked the infarct-limiting effect of preconditioning in rabbit heart *in vivo*. However, they were unable to abolish the protective effect of preconditioning with icatibant in an isolated buffer-perfused rabbit heart preparation even when the preconditioning stimulus was increased from one to four 5 min cycles of ischaemia. This apparent non-participation of bradykinin in preconditioning of the buffer-perfused heart was attributed to the lack of blood-borne kininogens. Similarly, Bugge & Ytrehus (1996) found that icatibant did not block the protective effects of preconditioning in an isolated rat heart preparation. However, Bouchard *et al*. (1998) found that preconditioning of isolated rat heart attenuated post-ischaemic endothelial dysfunction. This effect was not abolished by icatibant but was reversed by Lys [Leu^8] Des-Arg^9-bradykinin, a bradykinin B_1 receptor antagonist.

Despite these inconsistencies in the literature examining the participation of endogenous bradykinin in isolated heart preparations, the ability of exogenously administered bradykinin to mimic ischaemic preconditioning has been confirmed by numerous investigators in a variety of models. These include the isolated rabbit heart with infarct size as the endpoint (Goto *et al*., 1995); the isolated rat heart with infarct size (Goto *et al*., 1995; Bugge & Ytrehus, 1996; Starkopf *et al*., 1997; Feng & Rosenkranz, 1999; Feng *et al*., 2000; Ebrahim *et al*., 2000) and ischaemia-reperfusion arrhythmias (Hassanabad *et al*., 1998) as endpoints; in pigs subjected to infarction (Schulz *et al*., 1998); in isolated human cardiac tissue subjected to hypoxia and reoxygenation (Brew *et al*., 1995) and in humans undergoing coronary angioplasty with ST segment shift as the endpoint (Leesar *et al*., 1999).

Further evidence implying a central role for bradykinin in ischaemic preconditioning comes from mice with a targeted disruption of the bradykinin B_2 receptor gene. Yang *et al.* (1997b), using B_2 receptor knock-out mice, showed that ischaemic preconditioning did not confer protection against infarct size in these animals. These workers also demonstrated that rats deficient in high molecular weight kininogen did not display the preconditioning response (Yang *et al.*, 1997b). A recent study provides compelling genetic evidence supporting a cytoprotective role of bradykinin in the ischaemic heart (Yoshida *et al.*, 2000). The human tissue kallikrein gene was delivered into rats using adenoviral vector. One week following gene delivery, cardiac kinin levels were significantly increased. Hearts were subjected to coronary artery occlusion and reperfusion. It was observed that kallikrein gene delivery was associated with significant limitation of infarct size and attenuated severity of ventricular fibrillation. Finally, kallikrein gene delivery also attenuated apoptosis in the ischaemic zone, determined by terminal deoxynucleotidyl transferase-mediated nick end labelling. All the beneficial effects kallikrein overexpression were abolished by icatibant, implying a role for the bradykinin B_2 receptor in the protection observed.

Although the majority of work implicates involvement of bradykinin B_2 receptor activation in mediating the cardioprotective actions of bradykinin during ischaemia-reperfusion, there is limited evidence implying a role for bradkinin B_1 receptor activation. The bradykinin B_1 receptor is not constitutively expressed in most tissues but is inducible under certain pathological conditions such as inflammation and anoxia (Bhoola *et al.*, 1992). Activation of this receptor subtype has been proposed to mediate the vascular protection afforded by preconditioning. Bouchard *et al.* (1998) reported that the beneficial effects of ischaemic preconditioning on endothelial function was partly mediated by activation of the bradykinin B_1 receptor. In addition to this, Chahine and colleagues found that bradykinin limited noradrenaline outflow and reduced the occurrence of arrhythmias in the isolated rat heart model (Chahine *et al.*, 1993). This protective effect was not abrogated using Hoe 140 but with a specific bradykinin B_1 receptor antagonist, Lys [Leu^8] Des-Arg^9-bradykinin, implying a role for the bradykinin B_1 receptor as opposed to the bradykinin B_2 receptor (Chahine *et al.*, 1993).

Molecular mechanisms of bradykinin-induced acute cardioprotection

The mechanisms underlying the acute protective actions of bradykinin protection are not well understood. A number of agents have been proposed to participate in the protection including NO, prostaglandin I_2, protein kinase C (PKC) and tyrosine kinases (Vegh *et al.*, 1993; Goto *et al.*, 1995; Zhu *et al.*, 1995; Bugge & Ytrehus, 1996; Feng & Rosenkranz, 1999; Feng *et al.*, 2000). There is almost universal consensus that B_2 receptor activation is required for protection, since icatibant in most models abolishes the protection afforded by bradykinin. PKC activation is thought to be central in the preconditioning phenomenon and it has been proposed that once activated, it determines the phosphorylation of distal kinase and end effector proteins. Brew *et al.* (1995), Bugge & Ytrehus (1996) and Goto *et al.* (1995) have presented evidence that exogenously administered bradykinin protects against ischaemia-reperfusion injury through a PKC-dependent mechanism in isolated rat and isolated rabbit myocardium.

The role of NO in mediating the acute cardioprotective properties of both endogenous and exogenously administered bradykinin has been the subject of some interest. NO has been implicated in some studies as a mediator of the early cardioprotective actions of bradykinin (Schoelkens & Linz, 1992; Végh *et al.*, 1993; Zhu *et al.*, 1995; Feng *et al.*, 2000). However, in contrast to these studies, Goto *et al.* (1995) found that bradykinin-induced infarct limitaion in rabbit heart was not abrogated by L-nitroarginine methyl ester (L-NAME). Similarly Bugge & Ytrehus (1996) found that bradykinin-induced acute cardioprotection in rat heart was not modified by L-nitro-ω-arginine, a NO synthase (NOS) inhibitor with a similar pharmacological profile to L-NAME. Thus, there may be important species and end-point variations in the role of NO in mediating the acute cardioprotective actions of bradykinin, with some models showing NO dependency. As we shall see subsequently, although the acute infarct-limiting effect of bradykinin does not appear to be NO-dependent, the delayed effect of bradykinin may be mediated by NO generation.

Opening of the mitochondrial K_{ATP} channel has been proposed as distal mediator of preconditioning (O'Rourke, 2000) (see Figure 1). At present it is not known how opening of this channel might beneficially influence outcome from ischaemia. Some pharmacological evidence supports the notion that bradykinin may elicit protection through a mechanism involving mitochondrial K_{ATP} channel opening (Kita *et al.*, 2000; Sanada *et al.*, 2000). The mechanism by which this might occur is not clear. Several possibilities have been proposed including (i) the release of prostanoids, (ii) the generation of NO and (iii) the activation of a kinase cascade, downstream of PKC (as shown in Figure 1). By activating PLA_2 bradykinin stimulates production of prostaglandins. Although various prostanoids have been shown to activate sarcolemmal K_{ATP} channels, which have also been suggested to participate in classical preconditioning (Bouchard *et al.*, 1994; Sanada *et al.*, 2000), there is little evidence at present to suggest that prostanoids influence mitochondrial K_{ATP} channel opening. Similarly, it is not clear to what extent NO generated as a result of bradykinin B_2 receptor activation on endothelium participates in or contributes to mitochondrial K_{ATP} channel activation. There is some evidence that NO directly modulates mitochondrial K_{ATP} channel opening in cardiac myocytes (Sasaki *et al.*, 2000)

Endogenous bradykinin in relation to other endogenous mediators: the 'threshold' hypothesis of preconditioning

An important observation made by Goto *et al.* (1995) was the 'dose-dependency' of bradykinin's involvement in preconditioning. Anaesthetized, open chest rabbits were subjected to coronary artery occlusion with infarct size as the endpoint of protection. If three 5 min cycles of ischaemia were used to elicit the preconditioning response then icatibant did not block protection. However, if one 5 min cycle of

ischaemia was used to precondition the myocardium then protection was abrogated by icatibant. These workers, who had previously provided extensive evidence for the involvement of adenosine in ischaemic preconditioning, concluded that a 'threshold' must be reached in order for the full protective response of preconditioning to occur. It was proposed that when only one brief cycle of ischaemia is used, then bradykinin plays a primary role in inducing protection such that B_2 receptor blockade abolishes the effect. However, if three cycles are employed, other mediators are generated in sufficient quantity, such that the 'threshold' can be attained even in the presence of the B_2 receptor antagonist (see Figure 3). Experimental support for this important 'threshold hypothesis' has been provided by several investigators in relation to the use of ACE inhibitor drugs which is discussed in more detail below.

ACE inhibitors and the ischaemic myocardium

The clearly-defined role of ACE as a kininase of primary importance is supported by studies in which ACE inhibitors have been shown to elevate circulating and tissue bradykinin concentrations (Baumgarten *et al*., 1993; Pellacani *et al*., 1994; Hornig & Drexler, 1997). Baumgarten *et al*. (1993) showed that ramiprilat increased bradykinin outflow from the isolated ischaemic rat heart. This study showed that a local kallikrein system exists in the rat heart but additionally suggests that ACE inhibitors are capable of increasing bradykinin levels by inhibiting its breakdown (see Figure 4). With this in mind, several studies indicate that ACE inhibitors can potentiate a sub-threshold preconditioning stimulus by increasing bradykinin levels (Miki *et al*., 1996; Morris & Yellon, 1997; Ebrahim *et al*., 2001a). A subthreshold preconditioning stimulus is regarded as a short ischaemic period which liberates some or all of the triggers involved in classical preconditioning (including bradykinin) but their concentration is insufficient to trigger the protective response (see Figure 3). Miki *et al*. (1996) showed that captopril, combined with a subthreshold preconditioning protocol was sufficient to elicit the full preconditioning response in the open-chest rabbit coronary artery occlusion preparation, which was abrogated with icatibant, implying a role for the bradykinin B_2 receptor. Similarly, Morris & Yellon (1997) found that both captopril and lisinopril were able to enhance subthreshold preconditioning by augmenting bradykinin levels, an effect also eliminated with icatibant. More recently Ebrahim *et al*. (2001a) have shown that captopril enhanced a subthreshold preconditioning to induce marked limitation of infarct size in the isolated rat heart.

The ability of ACE inhibitors to confer protection in the absence of a preconditioning stimulus is more controversial (Heusch *et al*., 1997). In the studies previously described, administration of either captopril (Miki *et al*., 1996; Morris & Yellon, 1997; Ebrahim *et al*., 2001a) or lisinopril (Morris & Yellon, 1997) alone prior to the index ischaemic event did not

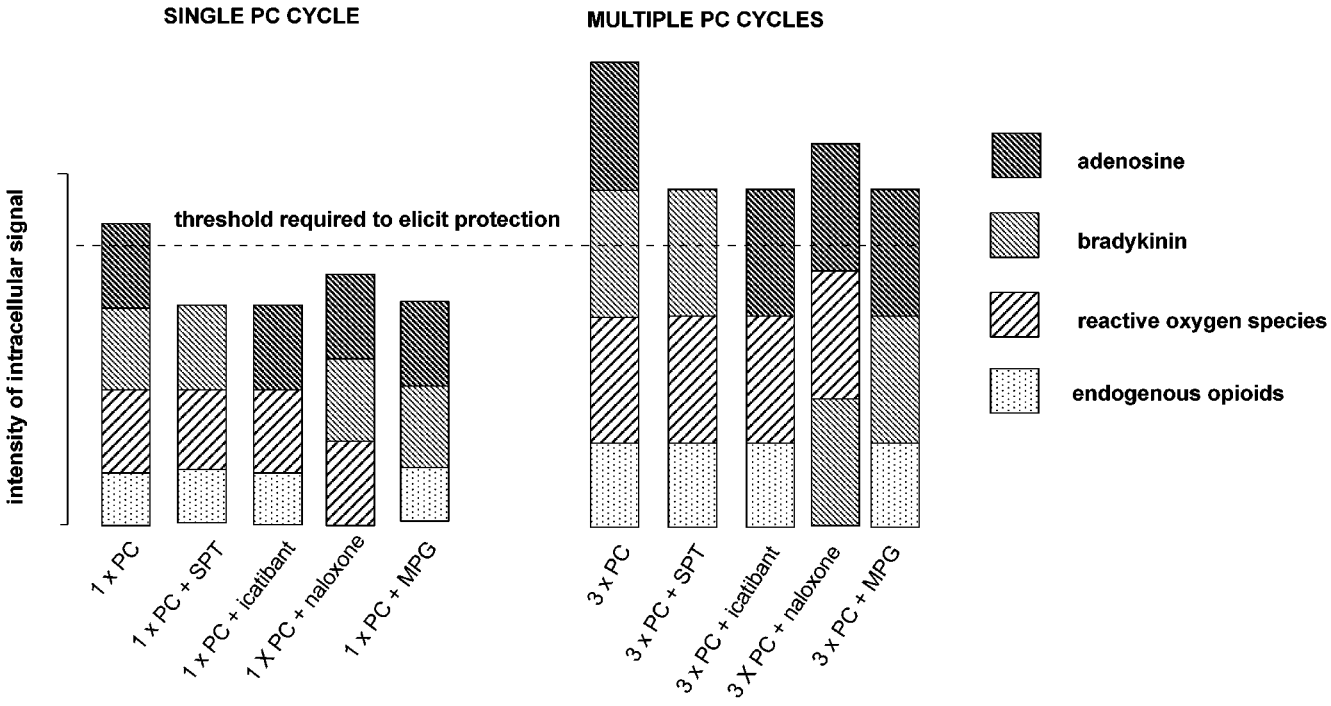

Figure 3 Schematic illustrating the multiple trigger hypothesis of classical preconditioning advanced by Goto *et al*. (1995). The hypothesis proposes that the preconditioning response is only elicited when a critical threshold of intracellular kinase activity is exceeded. All of the endogenous mediators known to act as triggers of the preconditioning response can independently activate the intracellular signal cascade but they act in concert to trigger the preconditioning response. When a single preconditioning cycle is used ($1 \times$PC), blockade of adenosine receptors with 8-sulphophenyltheophylin (SPT), blockade of bradykinin B2 receptors with icatibant, blockade of opioid receptors with (−)-naloxone or scavenging of free radicals with mercaptoprionylglycine (MPG) will be sufficient to reduce the intensity of the intracellular signal below the critical threshold. When multiple preconditioning cycles are used (e.g. $3 \times$PC), relatively more of the endogenous triggers are released. Under these conditions, antagonism of any single trigger may still result in sufficient kinase activation to exceed the threshold which elicits protection.

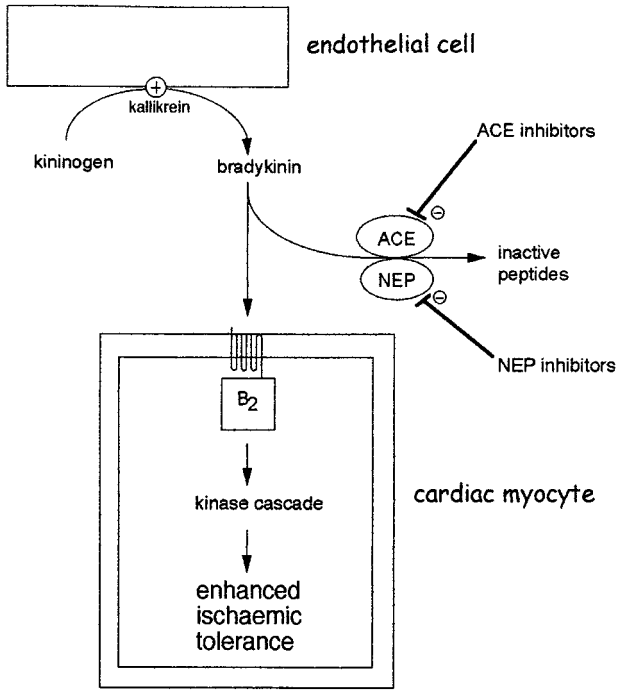

Figure 4 Augmentation of the preconditioning response by angiotensin converting enzyme (ACE) inhibitors or neutral endopeptidase (NEP) inhibitors. Bradykinin is efficiently and rapidly degraded by several enzymes especially ACE and NEP. Inhibition of these enzymes increases the availability of bradykinin at B2 receptors on cardiac myocytes. During very brief periods of ischaemia, interstitial bradykinin concentration may be insufficient to initiate the preconditioning mechanism. However, in the presence of an ACE or NEP inhibitor, augmentation of bradykinin concentration is sufficient to initiate preconditioning. The ability of ACE inhibitors to potentiate subthreshold ischaemic stimuli has been demonstrated for both early and delayed forms of preconditioning (see text).

result in protection. Those findings are in contrast to several other studies in which ACE inhibitor treatment alone was shown to protect against ischaemia-reperfusion injury (Ertl *et al*., 1982; Massoudy *et al*., 1994; Anderson *et al*., 1996; Dogan *et al*., 1998; Matoba *et al*., 1999; Jin & Chen, 2000; Weidenbach *et al*., 2000). The reasons for the lack of effect of ACE inhibitors in some studies are not clear. Anderson *et al*. (1996) showed that captopril but not enalapril was protective in the isolated rat heart and attenuated lipid peroxidation. Indeed, it has been proposed that ACE inhibitors, such as captopril, that possess sulfhydryl moieties are able to act as scavengers or reactive oxygen species and as a consequence are protective when administered alone (Anderson *et al*., 1996). However, the picture is complicated by the fact that some ACE inhibitors that do not possess a sulphydryl group have been reported to be cardioprotective in some models (Birincioglu *et al*., 1997; Matoba *et al*., 1999). Matoba *et al*. (1999) showed that cilazaprilat, a non-sulfhydryl containing ACE inhibitor, protected directly against hypoxia-reoxygenation injury in cultured rat myocytes and enhanced bradykinin production in the culture media of the myocytes.

The question of whether ACE inhibitors are independently cytoprotective in experimental acute myocardial ischemia without preconditioning remains unanswered. However, the recent Heart Outcomes Prevention Evaluation (HOPE) trial demonstrated that ramipril reduced risk of death in patients with coronary artery disease (HOPE Investigators, 2000), an effect that appears to be unrelated to blood pressure reduction alone.

Neutral endopeptidase inhibition

Of the several enzymes other than ACE that contribute to the inactivation of bradykinin, NEP is probably the most important (Ura *et al*., 1987; Piedimonte *et al*., 1994; Kokkonen *et al*., 1999). NEP, like ACE, is a cell surface zinc metalloprotease, but unlike ACE its concentration in endothelium is low. NEP is highly concentrated in the epithelial cells of the kidney, it is also found in lung, liver and myocardium (Bhoola *et al*., 1992; Piedimonte *et al*., 1994). Studies with NEP inhibitors have found that these agents can evoke cardioprotection (Schriefer *et al*., 1996; Yang *et al*., 1997a). Yang *et al*. (1997a) in an *in vivo* rat model of coronary artery occlusion found that the NEP inhibitor CGS24592 was able to induce cardioprotection comparable to that induced by an ACE inhibitor using infarct size as an end point.

Dual inhibitors of ACE and NEP are novel compounds often referred to as 'vasopeptidase inhibitors', a colloquial marketing term with no scientific provenance. They have been developed recently for the treatment of hypertension and heart failure (Fink *et al*., 1996; Robl *et al*., 1997; Weber, 1999; Asher & Naftilan, 2000; van Veldhuisen & van Gilst, 2000). Omapatrilat (BMS 186716) is the first in this new class of agents. In isolated rat hearts, using infarct size as an experimental end point, Ebrahim *et al*. (2001a) found that omapatrilat could potentiate a sub-threshold preconditioning stimulus sufficiently to evoke cardioprotection, which was also abrogated with icatibant. Interestingly, omapatrilat administered alone, provided moderate but significant cardioprotection, an effect also abrogated with icatibant. Ebrahim *et al*. (2001a) compared the effects of omapatrilat with a conventional ACE inhibitor, captopril. Although they found that captopril was able to enhance the sub-threshold preconditioning stimulus, similar to omapatrilat, when administered alone it was not cardioprotective. This implies that dual inhibition of ACE and NEP may have additional cardiovascular benefits when compared with ACE inhibition alone.

Rastegar *et al*. (2000a) reported that a dual ACE and NEP inhibitor, Z13752A, produced a protective effect in an *in vivo* dog model of coronary artery occlusion, using arrhthymia prevalence as an end point. They also found icatibant abolished the cardioprotective effect of Z13752A. Additionally, Schriefer *et al*. (1996) in an anaesthestized, open-chest rabbit model of coronary artery occlusion found that dual inhibition of ACE and NEP produced cardioprotective effects over and above treatment with just an ACE or NEP inhibitor alone. As these beneficial effects were blocked with icatibant, bradykinin-mediated cardiprotection is most likely. Since NEP is also responsible for the catalytic degradation of various other vasodilator peptides including atrial natriuretic peptide (ANP), type-B natriuretic peptide (BNP), type-C natriuretic peptide (CNP) and substance P (Piedimonte *et al*., 1994; Ozaki *et al*., 1999), it is feasible that any of these other vasodilator peptides may be involved in the cardioprotective effect observed with NEP inhibitors. ANP has been shown to

exert cardioprotective effects during ischaemia-reperfusion although these studies are equivocal. In one study (Rastegar *et al*., 2000b) ANP treatment was found to reduce ischaemia-reperfusion arrhythmias in canine heart. In another canine study (Takagi *et al*., 2000), no reduction in arrhythmias was observed but infarction was substantially limited by ANP treatment. BNP has been reported recently to limit infarct size in a concentration dependent manner in rat heart (D'Souza *et al*., 2002). However, the contribution of natriuretic peptides to the cardioprotective effects of ACE and NEP inhibitors appears to be overwhelmed by the contribution of bradykinin. Yang *et al*. (1997a) also reported that all protection afforded by a NEP inhibitor was abrogated using icatibant, implying a role only for bradykinin and not any of the other peptides augmented as a consequence of NEP inhibition. In addition, Yang *et al*. (1997a) used a selective natriuretic peptide receptor antagonist and were not able to abrogate the protection afforded by dual ACE and NEP inhibition, although it was slightly attenuated. Rastegar *et al*. (2000a) and Ebrahim *et al*. (2001a) found that the protection afforded by dual ACE/NEP inhibitors was lost in the presence of icatibant, strongly implicating a role for bradykinin rather than the other peptides.

Aminopeptidase P inhibition

Although ACE and NEP appear to play primary roles in bradykinin catabolism, recent reports imply that aminopeptidase P may be an important contributor to endogenous bradykinin turnover. The aminopeptidase inhibitor, apstatin, which has little affinity for ACE or NEP, was shown to be cardioprotective against ischaemia-reperfusion injury in isolated rat heart (Ersahin *et al*., 1999) and to limit infarct size in rat heart *in vivo* (Wolfrum *et al*., 2001). In both models, the protection afforded by apstatin was comparable to that seen with a selective ACE inhibitor and appeared to be bradykinin-mediated since icatibant abolished the protective properties of apstatin.

Bradykinin and delayed myocardial protection

Preconditioning the myocardium with ischaemia induces protection in two distinct phases; an early phase, which occurs immediately following the preconditioning stimulus and lasts for up to 2 h (classical preconditioning) and a late phase which occurs 24 h following a preconditioning stimulus and lasts for up to 72 h (Bolli, 2000; Baxter & Ferdinandy, 2001). Molecular triggers of classical preconditioning including adenosine, opioid peptides, and catecholamines have all been shown to also induce a delayed preconditioning-like effect (Baxter *et al*., 1994; Fryer *et al*., 1999; Meng *et al*., 1999). Preliminary evidence that bradykinin might act as a trigger of pacing-induced delayed protection against ischaemia-reperfusion arrhythmias was reviewed by Parratt *et al*. (1997). A role for a bradykinin in eliciting delayed protection against infarction has been established in two recent studies. Ebrahim *et al*. (2001b) administered 40 μg kg^{-1} bradykinin intravenously to rats and 24 h later studied responses to ischaemia-reperfusion. Using infarct size as the experimental end point, it was shown that hearts from bradykinin pretreated animals exhibited smaller infarct size and tendency towards better coronary flow (Ebrahim *et al*., 2001b). Kositprapa *et al*. (2001) showed that delayed ischaemic preconditioning against infarction in rabbit heart was abolished when icatibant was administered during the preconditioning stimulus. Conversely intra-atrial infusion of bradykinin (50 μg kg^{-1} min^{-1} for 15 min) resulted in significant limitation of infarction during coronary occlusion 24 h later. Interestingly, Ebrahim *et al*. (2001b) found that this late protective effect of bradykinin treatment was completely abrogated when bradykinin was administered following a NOS inhibitor, L-NAME (see Figure 5). This finding is compliant with the hypothesis proposed by Bolli and colleagues who have provided persuasive evidence that NO acts as a trigger of delayed preconditioning (see Bolli, 2000 for review). Indeed, it seems plausible that bradykinin acts as a primary trigger of delayed preconditioning, and that this effect is mediated by generation of NO as a signalling intermediate (Figure 5).

Further evidence supporting the involvement of bradykinin as a trigger of delayed preconditioning comes from recent work with the ACE inhibitor perindoprilat. Jaberansari *et al*. (2001) have reported that pretreatment of pigs with perindoprilat, potentiated a sub-threshold preconditioning stimulus (two 2 min coronary artery occlusions) sufficiently

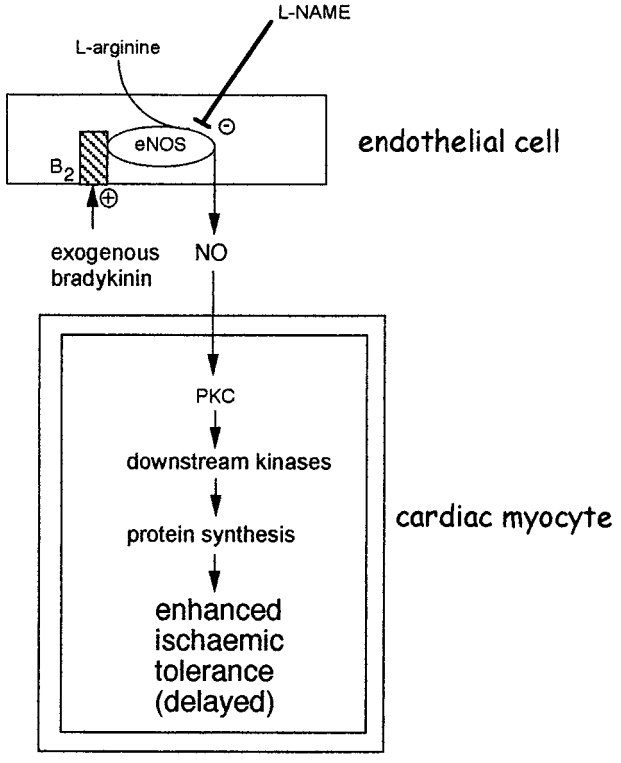

Figure 5 Proposed mechanism for the induction of delayed cardioprotection by bradykinin. The immediate activation of NOS as a result of bradykinin B_2 receptor activation leads to the generation of NO. The most likely NOS isoform is eNOS. According to prevailing the delayed preconditioning hypothesis, NO could subsequently trigger an adaptive response in cardiac myocytes, involving the activation of protein kinase C (PKC) isoforms and other kinases. The subsequent induction of unknown mediators of protection results in enhanced tolerance to ischaemia 24 h following liberation or application of bradykinin. Adapted from Ebrahim *et al*. (2001b).

to induce delayed preconditioning comparable to that induced by four 5 min coronary occlusions. Although this study does not provide direct evidence for the involvement of bradykinin, the result was compatible with the hypothesis that bradykinin (or other peptides catalytically inactivated by ACE) might be implicated in triggering the delayed phase of preconditioning. As with other ACE-inhibitor studies, this demonstration of an association with delayed preconditioning may have important implications for our perceptions of ACE inhibitors as cardioprotective agents.

Bradykinin, apoptosis and attenuation of reperfusion injury

The majority of cardioprotective agents including bradykinin have to be administered prior to the ischaemic insult in order to limit injury. However, as it is difficult to predict when most patients are likely to encounter an ischaemic event, it is rarely feasible to administer these agents to patients. Intermittent administration to high-risk patients such as those with unstable angina is feasible. Greatest benefit in the clinic would be observed if agents could be given at reperfusion and thus limit reperfusion injury. Ischaemia / reperfusion injury results in necrosis and apoptosis. While, reperfusion of the jeopardized myocardium is imperative, reperfusion itself can result in irreversible cell injury, either through necrosis and/or apoptosis (Yellon & Baxter, 1999). Most agents that limit ischaemia-reperfusion injury must be administered prior to the onset of ischaemia insult to be effective. However, some agents may influence the the apoptotic programme which is activated or enhanced during reperfusion. Inhibitors of the apoptotic cascade can be administered at the onset of reperfusion or just prior to the onset of reperfusion (Mocanu *et al*., 2000). Peptide growth factors such as transforming growth factor-β_1, insulin-like growth factor, insulin, cardiotrophin and fibroblast growth factors limit reperfusion injury at least partially through activation of anti-apoptotic 'survival' signal pathways. These include phosphatidyl inositide 3′-OH kinase (PI3 kinase), Akt/protein kinase B (PKB) and p42/p44 mitogen activated protein kinases (Yellon & Baxter, 1999). Recent evidence has demonstrated that bradykinin can activate PI3 kinase in myocytes (Ritchie *et al*., 1999). Hence, it appears feasible that bradykinin may limit reperfusion injury by activating at least one 'survival' kinase pathway. Massoudy *et al.* (1994) demonstrated that bradykinin and or ramiprilat administered at reperfusion improved recovery of mechanical function in a guinea pig isolated heart preparation. In studies by Yang *et al.* (1997a; 1999) and by Shrieffer *et al.* (1996), ACE inhibitors, NEP inhibitors or dual ACE/NEP inhibitors were cardioprotective when given at reperfusion. However, in eNOS knockout mice, the cardioprotective of an ACE inhibitor at reperfusion was absent, suggesting that the protection afforded by enhanced bradykinin at reperfusion was NO-dependent (Yang *et al*., 1999). The bradykinin B_2 receptor is coupled to eNOS and in the presence of bradykinin, eNOS is uncoupled leading to its activation (Marrero *et al*., 1999). We have observed that bradykinin administered at reperfusion in an isolated rat heart model limited infarct size, an effect abrogated by an inhibitor of PI3 kinase, wortmannin (unpublished data). Thus, bradykinin may be effective in attenuating reperfusion injury, which in turn implies that agents that increase bradykinin levels, such as ACE inhibitors, could theoretically be applied to limit reperfusion injury.

Conclusion and future perspectives

Bradykinin exerts a unique and robust pattern of injury limiting actions in the ischaemic and reperfused heart. A wealth of evidence suggests that bradykinin administered prior to the onset of myocardial ischaemia exerts a cardioprotective effect in animal and human experimental models. Endogenous bradykinin participates in classical preconditioning in many experimental models. Recently, a potential role for bradykinin in eliciting a delayed phase of preconditioning has emerged. Bradykinin has also been shown to limit reperfusion injury, an action which may be beneficial in patients receiving reperfusion therapy for acute myocardial infarction. Of current therapeutic relevance, agents that inhibit the breakdown of bradykinin, notably ACE inhibitors and the newly introduced combined ACE/NEP inhibitors, may display valuable protective effects both experimentally and clinically. Thus, administration of exogenous bradykinin in some clinical settings and augmentation of endogenous bradykinin levels may soon become feasible and valuable approaches to the treatment of ischaemic heart disease.

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