Inhibitors of Bradykinin-Inactivating Enzymes Decrease Myocardial Ischemia/Reperfusion Injury following 3 and 7 Days of Reperfusion

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ABSTRACT

Inhibitors of bradykinin (BK)-inactivating enzymes protect from myocardial ischemia/reperfusion injury after short periods of reperfusion. However, protection after 2 to 3 h of reperfusion does not mean that myocardium remains viable for an extended time. Therefore, we examined the effects of inhibitors of angiotensin-converting enzyme (ramiprilat), EP24.11 (cFP-F-pAB), and EP24.15 (cFP-AAF-pAB) in a chronic model of myocardial ischemia/reperfusion injury. A left descending coronary artery was occluded for 30 min in anesthetized rabbits. Saline, ramiprilat, or endopeptidase inhibitors were given after 27 min of occlusion. The BK$_2$ receptor antagonist HOE1410 was administered in certain experiments. After ischemia, the occlusion was removed, and the animal allowed to recover for 3 or 7 days. Surgery was then repeated, and the heart removed for determination of infarct size. In separate experiments, the heart was removed after 2 h of reperfusion for determination of BK tissue levels. Ramiprilat and endopeptidase inhibitors reduced infarct size at 3 and 7 days. Combining inhibitors further reduced infarct size after 3 days. The protective effect of the endopeptidase inhibitors was blocked by HOE140. Infarct sizes at 7 days were larger than at 3 days. The additive effect of multiple inhibitors was absent at 7 days. Ramiprilat and cFP-F-pAB significantly increased tissue BK levels. We conclude that inhibition of BK-inactivating enzymes protects endogenous BK from degradation and provides long-lasting protection from myocardial ischemia/reperfusion injury. A single treatment at the time of reperfusion does not prevent extension of the infarction between 3 and 7 days.

Myocardial ischemia and subsequent reperfusion of the ischemic tissue has been associated with various types of injury, including lethal reperfusion injury, myocardial stunning, and cardiac arrhythmias. Preventing or reducing these myocardial ischemia/reperfusion injuries could improve the beneficial effects of therapies, such as coronary artery by-pass grafting, angioplasty, and thrombolytic agents, intended to cause reperfusion of ischemic tissue (Hearse and Bolli, 1992). ACE inhibitors have been shown to reduce myocardial ischemia/reperfusion injury. This protective effect is produced through an action to preserve the peptide BK from rapid degradation, rather than by decreasing production of angiotensin (ANG) II (Martorana et al., 1990; Hartman et al., 1993a, b). BK is generally considered to be cardioprotective, and is released in the heart during ischemia, but its action is limited by rapid local inactivation by a variety of enzymes present in the heart, including ACE (Linz et al., 1996). The BK-inactivating endopeptidases EP24.11 and EP24.15 are also present in the heart (Schriefer et al., 1996) and could degrade BK when ACE is inhibited. Previous work in our laboratory has demonstrated that inhibitors of ACE, EP24.11 and EP24.15, reduced myocardial ischemia/reperfusion injury in an in vivo rabbit model using 30 min of regional ischemia and 2 h of reperfusion (Schriefer et al., 1996). Combinations of enzyme inhibitors had additive effects. The cardioprotective effects of the inhibitors were mediated by BK$_2$ receptors. Like ACE inhibitors, the endopeptidase inhibitors appear to act by preserving endogenous BK from metabolism. There is, however, no published information regarding the effects of ACE or endopeptidase inhibitors beyond this short 2-h reperfusion period. The question remains whether these inhibitors prevent ischemia/reperfusion injury or merely delay it for a short time. To address this question, we examined the effects of the specific endopeptidase inhibitors cFP-F-pAB and cFP-AAF-pAB (Almenoff and Orlowski, 1983; Orlowski

et al., 1988) and the ACE inhibitor ramiprilat on ischemia/reperfusion injury in an in vivo rabbit model with a more clinically significant reperfusion period of 3 or 7 days.

**Experimental Procedures**

**Materials.** Ramiprilat, the active form of the prodrug ramipril, was a gift of Pharmacia (Peapack, NJ). The inhibitors of EP24.11 and EP24.15, cFP-F-pAB and cFP-AAF-pAB, respectively, were synthesized in our laboratory as previously described (Schriefer and Molin- eaux, 1993). The EP24.15 inhibitor cFP-AAF-pAB is converted in vivo to a potent ACE inhibitor by the action of EP24.11 (Williams et al., 1993). Thus, cFP-AAF-pAB was only used in combination with the EP24.11 inhibitor to prevent this conversion (Cordoze and Or- lowski, 1993). Pilot studies demonstrated that the doses of cFP-F- pAB and cFP-AAF-pAB used (2 μmol/kg for each) provided maximal protection from ischemia/reperfusion injury. Pilot studies also demonstrated that the dose of ramiprilat used in this study (50 μg/kg) provided maximal protection from ischemia/reperfusion injury. Neither larger doses of ramiprilat nor addition of enalaprilat to ramipril- lat provided better protection. HOE140 was a gift of Hoechst-Roussel Pharmaceuticals (now Aventis, Strausbourg, France). Other drugs and chemicals were obtained from Sigma-Aldrich Chemical (St. Louis, MO).

**Animals.** All animal procedures in this investigation were in compliance with the Animal Welfare Act Regulations, 9 CFR Parts 1, 2, and 3, with the Guide for Care and Use of Laboratory Animals (Department of Health, Education, and Welfare, 1985) and with the Declaration of Helsinki. Male and female New Zealand White rabbits (Prince’s Rab- bitry, Oak Hill, WV), weighing 2.5 to 3.5 kg, were used in all experiments and were randomly assigned to the treatment groups.

**Rabbit Model of Ischemia/Reperfusion.** Rabbits were anes- thetized with ketamine (35 mg/kg i.m.) and xylazine (5 mg/kg i.m.). Anesthetized animals were intubated and artificially ventilated with a mixture of 100% oxygen and room air. Respiration was adjusted to maintain blood pH in the physiological range. Anesthesia was main- tained by i.v. infusion of xylazine (2 mg/ml at 0.3–0.4 ml/min) via a lateral ear vein and by i.m. injections of 0.4 ml of a solution of ketamine (80 mg/ml) and xylazine (5 mg/ml) at 30-min intervals. Electrodes were placed on the limbs for recording of ECG. Rectal temperature was monitored by a telethermometer probe and body temperature maintained at 39°C with a heating pad. A left lateral thoracotomy was performed through the 4th intercostal space. A 4-0 prolene suture was passed through the pericardium and myocar- dium beneath and around a major left ventricular descending coronary artery and through a short length of polyethylene tubing to create a reversible snare occluder. Tightening the snare occluded the artery and produced regional ischemia. The suture was placed in such a location as to produce an ischemic area of approximately 50% of left ventricular mass. Experimental drugs were administered after 27 min of occlusion via a lateral ear vein. Ramiprilat was adminis- tered at a dose of 50 μg/kg; cFP-F-pAB and cFP-AAF-pAB were administered at a dose of 2 μmol/kg. At these doses, the drugs, singly or in combinations, produced no alterations in systemic blood pressure or heart rate (Schriefer et al., 1996), thus any cardioprotection seen with the treatments is not likely to be due to changes in hemodynamic status. The coronary artery snare was released after 30 min, but the suture was left in place. The chest was closed in layers and the animals allowed to recover. Postsurgical rabbits re- ceived enrofloxacin (10 mg/kg i.m., every 24 h) as prophylaxis against infection, and buprenorphine (20 μg/kg s.c., every 12 h for 24 h) for analgesia. After 3 or 7 days of reperfusion, the animals were anes- thetized as described above and the surgery was repeated to harvest the heart. The coronary artery was resected, and India Ink was injected directly into the left ventricle to delineate the normally perfused areas of the heart from the area perfused by the occluded artery (ischemic area). Simultaneously with the injection of India Ink, the rabbit was euthanized with an overdose of sodium pento- barbital (250 mg/kg i.v.). The heart was removed from the chest, rinsed, and sliced (2–3-mm sections) from the apex to the base, perpendicular to the long axis of the heart.

In a separate set of experiments, rabbits were treated with either saline, ramiprilat, or cFP-F-pAB and euthanized after 2 h of reper- fusion. Hearts were removed and the left ventricular freewall iso- lated and sectioned into area above the occlusion (normally perfused) or below the occlusion (ischemic area). The sections were frozen in liquid nitrogen and stored at −80°C for measurement of BK content.

**Determination of Myocardial Infarct Size.** Myocardial slices were incubated for 15 min at 37°C in triphenyl tetrazolium chloride (1% in phosphate-buffered saline). Salvaged myocardium stained brick red, while infarcted tissue remained gray. The outline of each slice (both sides) was traced on a transparency, with the normally perfused, salvaged, and infarcted areas indicated. The tissue slices were then weighed. The tracings of the slices and an image analysis system were used to determine the areas of the normally perfused, ischemic area (salvaged + infarcted), and infarcted myocardium. The respective areas for each side of each slice were averaged. Using those averages and the mass of each slice, normally perfused, isch- emic area, and infarct masses for each slice and the entire heart were then calculated. Analysis of infarct size was done in a blinded man- ner, since determination of color changes in the heart following staining was somewhat subjective.

**Measurement of BK Content of Left Ventricle.** Frozen tissues were homogenized in 4 M guanidine thiocyanate and 1% trifluoro- acetic acid. Homogenates were sonicated briefly and centrifuged at 5000g for 20 min at 4°C. BK concentration in the supernatants was determined by radioimmunoassay using a commercial kit (Peninsula Laboratories, Belmont, CA). Protein content of the supernatants was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA). BK content of the ventricle was expressed as picograms of BK per milligram of protein.

**Statistics.** Analysis of variance (ANOVA) was used to determine the statistical significance of time and treatment effects. Since the size of the ischemic area could influence infarct size, ischemic area was treated as a covariate in the analysis to determine whether differences in ischemic area contributed to apparent differences in infarct size. When statistically significant differences were indicated by ANOVA, post hoc testing with Duncan’s multiple range test was used to determine which group means were statistically different. The level of statistical significance for all tests was p < 0.05.

**Results**

**Ischemic Area.** There were no significant differences in the size of the ischemic area in any of the treatment groups at either 3 or 7 days of reperfusion (Figs. 1A and 2A). In addition, analysis of covariance showed that the size of the ischemic area was not responsible for the differences in infarct size, ischemic area, and infarct masses for each slice and the entire heart were then calculated. Analysis of infarct size was done in a blinded man- ner, since determination of color changes in the heart following staining was somewhat subjective.

**Examination of Infarct Sizes According to Gender.** Both male and female rabbits were used in this study. There were no significant gender differences in infarct size in control or treated rabbits (data not shown); thus male and female values were combined.

**Infarct Size following 3 Days of Reperfusion.** When administered alone, both ramiprilat and the EP24.11 inhibitor cFP-F-pAB significantly reduced infarct size, compared with saline (Fig. 1B). The addition of the EP24.15 inhibitor cFP-AAF-pAB to the EP24.11 inhibitor (EPI COMBO) re- sulted in a significant decrease in infarct size, compared with cFP-F-pAB alone. Combining ramiprilat and both endopep- tidase inhibitors (EPI COMBO + ramiprilat) produced a further significant reduction in infarct size. The protective
The effect of the EP inhibitors was blocked by the BK2 receptor antagonist HOE140, while HOE140 alone had no effect on infarct size.

Infarct Size following 7 Days of Reperfusion. As was the case with 3 days of reperfusion, both ramiprilat and the EP24.11 inhibitor cFP-F-pAB significantly reduced infarct size, compared with saline (Fig. 2B). However, combinations of enzyme inhibitors did not result in further significant decreases in infarct size. HOE140 alone had no effect on infarct size, but did block the protective effects of the enzyme inhibitors.

Comparison of Infarct Sizes following Differing Reperfusion Times. Infarct sizes tended to be larger when examined after 2 h of reperfusion in the same model (Schriefer et al., 1996). Infarct size for the 2-h reperfusion groups (data not shown) was not significantly different from that in the 3- or 7-day models. Infarct size at 2 h of reperfusion was similar to that at 3 days, but differed from that at 7 days in the EPI COMBO and EPI COMBO + ramiprilat groups.

Infarct sizes for 2 h, and 3 and 7 days of reperfusion were examined using a two-way analysis of variance with time of reperfusion and drug treatment as variables. Both the time and treatment effects were found to be significant; the interaction was not significant. Post hoc testing revealed that infarct sizes on day 7 were significantly greater than those for the day 3 and 2-h groups when treatment groups were collapsed. Post hoc testing also revealed that the EPI COMBO + ramiprilat treatment gave significantly better protection compared with all other treatments when time of reperfusion was collapsed.

BK Content of Left Ventricle. The BK content of the normally perfused area in cFP-F-pAB-treated animals was significantly higher than in saline-treated animals (Table 1). The BK content of the left ventricle was significantly higher.
Discussion

This study demonstrates that inhibitors of the BK-degrading enzymes EP24.11, EP24.15, and ACE attenuate myocardial ischemia/reperfusion injury for up to 7 days following a single administration at the time of reperfusion. These results extend previous observations (Hartman et al., 1993a; Schriefer et al., 1996) that these inhibitors reduce myocardial infarct sizes when examined after 2 h of reperfusion.

When given alone, ramiprilat and cFP-F-pAB significantly reduced infarct size at 3 and 7 days of reperfusion, indicating that a single administration of the drugs can provide long-lasting protection from myocardial damage produced by 30 min of regional ischemia followed by reperfusion. Combining the EP24.15 inhibitor cFP-AAF-pAB with cFP-F-pAB produced an additive protective effect at 3 days of reperfusion, an effect previously seen at 2 h of reperfusion (Schriefer et al., 1996). When inhibitors of all three enzymes were combined, a further additive effect was noted at 3 days, again similar to the effect previously seen at 2 h. Inhibition of a single enzyme, either ACE or EP24.11, did not produce the greatest possible protection from ischemia/reperfusion injury, perhaps because degradation of BK is shifted to other enzymes present in heart. Better protection was seen when multiple enzymes were inhibited to minimize this shift. Blais et al. (1997) reported that ACE is responsible for approximately 50% of BK metabolism in rabbit heart. Other enzymes, including EP24.11, EP24.15 (Schriefer et al., 1996; Yang et al., 1997; Raut et al., 1999), and aminopeptidase P (Ersahin et al., 1999), are presumably responsible for the remainder. A limitation of the present study is that the EP24.15 inhibitor used could not be used alone due to the fact that EP24.11 converts it to an ACE inhibitor (Williams et al., 1993). Thus, the contribution of EP24.15 to BK metabolism in the heart will require the use of an inhibitor that can be administered singly. The BK2 receptor antagonist HOE140 blocked the protective effects of the endopeptidase inhibitors and ramiprilat in this and previous studies (Hartman et al., 1993b; Schriefer et al., 1996), indicating that the cardioprotective effects of these enzyme inhibitors are mediated via BK2 receptors. The effect of ramiprilat in this model has previously been shown to be
independent of effect on ANG II synthesis, since the ANG II receptor antagonist losartan had no effect on the cardioprotective action of ramiprilat (Hartman et al., 1993a).

The EP24.15 inhibitor used in this study, cFP-AAF-pAB, is a specific, active site-directed inhibitor of the enzyme (Orlowski et al., 1988). The related compound cFP-F-pAB is a specific, active site-directed inhibitor of EP24.11 (Almenoff and Orlowski, 1983). cFP-F-pAB has no inhibitory effect on EP24.15 and cFP-AAF-pAB has no inhibitory effect on EP24.11 (Orlowski et al., 1983; Kest et al., 1992). The effect of the EP24.11 inhibitor cFP-F-pAB on a similar enzyme, endothelin-converting enzyme 1 (ECE-1), is unknown. ECE-1 converts big endothelin to endothelin-1. Endothelin-1 has been shown to suppress BK release in the isolated rat heart (Zeitlin et al., 1996), thus if cFP-F-pAB inhibited ECE-1, BK levels might increase due to an effect on endothelin rather than protection of BK from metabolism by EP24.11. Although EP24.11 and ECE-1 are similar enzymes, there are differences that make it unlikely that cFP-F-pAB would be active against ECE-1. Analysis of the active sites of EP24.11 and ECE-1 (Sansom et al., 1995) reveals differences in the structure between the enzymes that make it unlikely that the active site-directed cFP-F-pAB would bind to ECE-1. In addition, unlike EP24.11, ECE-1 exists as a disulfide-linked dimer and is not inhibited by other EP24.11 inhibitors such as thiorphan (Turner and Murphy, 1996). These differences make it unlikely that cFP-F-pAB exerts any of its cardioprotective effects by an action involving endothelin.

cFP-F-pAB and cFP-AAF-pAB have been shown to enhance the in vitro response to BK in the uterus (Schriever and Molineaux, 1993), and to protect the rabbit heart from ischemia/reperfusion injury and block EP24.11 suppression of BK (Schriever et al., 1999). The enzyme inhibitors used in this study could activate BK2 receptors indirectly by preserving endogenous BK, or by directly affecting the receptors. The heart contains a kallikrein-kinin system capable of producing BK locally (Nolly et al., 1994). BK is released from the ischemic heart (Kimura et al., 1973) and protects from ischemia/reperfusion injury (Martorana and Schölkens, 1992; Wall et al., 1994). Cardioprotection by endogenous BK would be limited by rapid, local enzymatic degradation. Blocking BK degradation increased BK-induced cardioprotection (Martorana et al., 1990; Hartman et al., 1993a,b; Schriever et al., 1996), and would be expected to increase concentrations of BK in the heart. Such an increase in BK concentrations in the hearts of animals treated with inhibitors of ACE and EP24.11 was demonstrated in the present study. Thus, it appears that the protective effect of the inhibitors resulted from decreased metabolism of endogenous BK.

While ACE inhibitors protect BK from metabolism and thereby potentiate the effects of endogenous BK, they may also increase BK actions by mechanisms that are independent of their ability to prevent enzymatic degradation of BK. In isolated guinea pig atria, the ACE inhibitor enalaprilat potentiated the positive inotropic effect of a BK analog that is not metabolized by ACE. In transfected Chinese hamster ovary cells exposed to enalaprilat, the ACE-resistant BK analog increased activation of BK2 receptors in cells that expressed ACE, but not in cells that did not express ACE (Erdös et al., 1999). The authors conclude from these studies that a combination of an ACE inhibitor and ACE increases the sensitivity of BK receptors to agonists. Ramiprilat has been reported to decrease sequestration of BK2 receptors following stimulation by BK in porcine endothelial cells and to reactivate signaling events initiated by stimulation of the receptor (Benzing et al., 1999). Another ACE inhibitor, captopril, has been shown to increase the affinity of BK2 receptors in bovine coronary artery endothelial cells (Miyamoto et al., 2000). Thus, it is possible that a portion of the cardioprotective effects of ramiprilat seen in the present study could be due to interactions with BK2 receptors, in addition to preserving BK from degradation. To our knowledge, there is no published information on the possibility that the endopeptidase inhibitors used in this study could have a similar interaction with BK2 receptors.

The mechanisms by which BK produces its cardioprotective action in myocardial ischemia/reperfusion injury are unclear. BK produces a number of effects that might be beneficial during ischemia/reperfusion, including increasing coronary perfusion, changing cardiac metabolism to preserve high-energy phosphates and increase glucose uptake, and reducing release of norepinephrine, and thus arrhythmias (Dendorfer et al., 1999). The beneficial effects of BK appear to be mediated by release of nitric oxide (NO) and, depending upon the species, prostaglandins or calcium-activated potassium channels (Dendorfer et al., 1999). In a rabbit model similar to the one used in this study, the cardioprotective effect of ramiprilat was blocked by the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (Hartman et al., 1994).

In the present study, enzyme inhibitors were not present during ischemia but were administered at the beginning of reperfusion. Thus, endogenous BK released during the ischemic period would be subject to rapid degradation, while BK released during reperfusion would be protected. Under these conditions, production of NO in response to BK would be increased during the reperfusion period but not the ischemic period. This is an important consideration, since NO can have a beneficial effect on the heart during reperfusion (Naikanishi et al., 1992) but a deleterious effect during ischemia (Araki et al., 2000).

A single administration of all three enzyme inhibitors given at the time of reperfusion reduced infarct size from approximately 30% of left ventricular mass to approximately 10% at 2 h and 3 days of reperfusion. It is unknown whether additional administrations of enzyme inhibitors during the 3-day reperfusion period would result in infarct sizes lower than 10%. It seems unlikely, however, since infarct sizes are the same at 2 h as at 3 days. It appears more likely that reduction of infarct sizes to approximately 10% represents the maximal effect on infarct size possible by this treatment. One limitation on the effectiveness of the treatment may involve alterations in ANG metabolism. ANG-(1-7) is produced from ANG I by the actions of EP24.11 and EP24.15. ANG-(1-7) production is increased by ACE inhibition due to decreased conversion of ANG I to ANG II. ANG-(1-7) is biologically active and has effects opposite to those of ANG II (Ferrario and Iyer, 1998). Simultaneous inhibition of ACE and EP24.11 reduced plasma ANG-(1-7) levels and increased ANG II levels in rats following myocardial infarction (Duncan et al., 1999). Decreased ANG-(1-7) may compromise the therapeutic effect of ACE inhibition since ANG-(1-7) contributes to some of the beneficial effects of ACE inhibitors on blood vessels (Li et al., 1997; Iyer et al., 1998). It is unknown
whether similar alterations in ANG-(1-7) occur when multiple enzymes are inhibited in the model used in the present study. However since ANG-(1-7) has been shown to potentiate BK effects (Paula et al., 1995; Gorelik et al., 1998), it is possible that simultaneous inhibition of ACE, EP24.11, and EP24.15 may decrease the effectiveness of this combination in limiting infarct size following ischemia/reperfusion by decreasing ANG-(1-7). Other processes, not sensitive to BK, may also be responsible for a portion of the ischemia/reperfusion injury produced in this model. One possibility for such a process is release of reactive oxygen species from leukocytes (Hearse and Bolli, 1992).

When treatment was eliminated as a variable, two-way ANOVA revealed that infarct size was significantly larger after 7 days of reperfusion compared with 2 h or 3 days. In addition, while individual inhibitors protected at 7 days, they tended to be less effective, and the additive effect of multiple enzyme inhibitors, seen at 2 h and 3 days, was lost. It may be that enzyme inhibitors given at reperfusion delay full development of the injury for 3 days, but that injury progresses after that time. An alternate explanation is that a late occurring process extended infarcts between 3 and 7 days of reperfusion, and that BK was less effective in limiting this late process. Infarcts following myocardial ischemia/reperfusion injury are produced by both necrosis (Hearse and Bolli, 1992) and apoptosis (Gottlieb et al., 1994). The differences in BK-induced cardioprotection at day 3 and day 7 might be explained if BK influenced these processes to a different extent. At the doses used in this study, the duration of biological effect of BK-induced cardioprotection at day 3 and day 7 might be explained if BK influenced these processes to a different extent. At the doses used in this study, the duration of biological effect of BK-induced cardioprotection at day 3 and day 7 might be explained if BK influenced these processes to a different extent.

We conclude that inhibition of BK degradation during reperfusion protected the heart from ischemia/reperfusion injury for up to 7 days. Maximal protection required inhibition of multiple BK-degrading enzymes. It is apparent that a single treatment at the time of reperfusion does not prevent a slight extension of the infarcted tissue between 3 and 7 days of reperfusion.

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References


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