Kallikrein-Kinin System

Angiotensin-Converting Enzyme Inhibition After Experimental Myocardial Infarct
Role of the Kinin B1 and B2 Receptors

Arvi Duka, Ekaterina Kintsurashvili, Irena Duka, Deborah Ona, Teresa A. Hopkins, Michael Bader, Irene Gavras, Haralambos Gavras

Abstract—We sought to define the contribution of each of the 2 kinin receptors (bradykinin 1 receptor [B1R] and bradykinin 2 receptor [B2R]) to the cardioprotection of angiotensin-converting enzyme (ACE) inhibition after acute myocardial infarct. Wild-type mice and gene knockout mice missing either B1R or B2R were submitted to coronary ligation with or without concurrent ACE inhibition and had evaluation of left ventricular systolic capacity by assessment of fractional shortening (FS). Baseline FS was similar in all of the animals and remained unchanged in sham-operated ones. At 3 weeks after myocardial infarct, in the wild-type group there was a 27% reduction of FS (P<0.5) without ACE inhibition and 8% with ACE inhibition; in the B1R−/− groups the FS was reduced by 24% and was no different (at 28%) with ACE inhibition; in the B2R−/− groups, however, the FS was decreased by 39% and with ACE inhibition was decreased further by 52%. Analysis of bradykinin receptor gene expression in hearts showed that when one receptor was missing, the other became significantly upregulated; but the B1R remained highly overexpressed in the B2R−/− mice throughout, whereas the overexpressed B2R became significantly suppressed in the B1R−/− mice in a manner quantitatively and directionally similar to that of wild-type mice. We conclude that both bradykinin receptors contribute to the cardioprotective bradykinin-mediated effect of ACE inhibition, not only the B2R as believed previously; but, whereas with potentiated bradykinin in the absence of B1R, the upregulation of B2R is simply insufficient to provide full cardioprotection, in the absence of B2R, the upregulated B1R actually seems to inflict further tissue damage. (Hypertension. 2008;51:1352-1357.)

Key Words: bradykinin receptors • myocardial ischemia • cardioprotection • ACE inhibition • gene knockout mice

The cardioprotective effect of angiotensin-converting enzyme (ACE) inhibition is now well established, but the multiple mechanisms contributing to this effect are still being investigated, and new aspects and functions of the ACE are being reported. The most important and clinically relevant results of ACE inhibition are diminished formation of angiotensin II and prolonged activity of bradykinin. However, whereas the former has been widely explored in studies ranging from basic research to large outcome clinical trials, the latter remains less well understood.

Earlier studies explored the physiopharmacology of bradykinin and defined the bradykinin receptor (BR) 1 (B1R) and 2 (B2R), which mediate their multiple hemodynamic and metabolic effects mainly via use of peptide analogs with agonistic (B2R), which mediate its multiple hemodynamic and metabolic actions of bradykinin are exerted via activation of the constitutive B2R. Indeed, acute or chronic infusion of B2R antagonists in animals or humans was shown to partly reverse the antihypertensive effect of ACE inhibitors, to prevent the cardioprotective action of ACE inhibition in animals submitted to cardiac ischemia/reperfusion injury, and to inhibit the amelioration of insulin-dependent glucose transport by ACE inhibitors. On the contrary, the B1R, of which the ligands are des-Arg metabolites of bradykinin, is believed to be mostly unexpressed under normal conditions but is highly inducible by lipopolysaccharides, bacterial toxins, and inflammatory mediators resulting from tissue injury.

Advances in molecular biology led to the cloning and characterization of these receptors and the creation of genetically engineered mice with deletion first of the B2R and, more recently, the B1R. The B2R knockout mice have been more extensively studied: They seem to have higher blood pressure (BP) at baseline and more salt-sensitive BP, they are less responsive to preconditioning-induced cardioprotection from ischemia/reperfusion injury, which would suggest an increased propensity to ischemic myocardial tissue damage.
damage; and they have less efficient myocardial metabolism with increased cardiac oxygen consumption. However, they have the same magnitude of hypotensive response to ACE inhibition as their wild-type (WT) counterparts. This seemingly paradoxical observation was explained by the subsequent finding that elimination of the B2R results in significant upregulation of the B1R, which can assume to a large extent the vasoreactive properties of the B2R. The B1R knockout mice appear to have a normal baseline cardiovascular phenotype, but their responses to cardiovascular dysregulation are less well explored.

The present experiments were designed to further investigate the contribution of each BR to cardioprotection after experimental myocardial infarction (MI) with or without treatment with ACE inhibition, by comparing WT mice with mice missing each one of these receptors, ie, B1R or B2R gene knockout mice.

Methods

Animals and Procedures

Male B1R gene knockout mice (B1R−/−), B2R gene knockout mice (B2R−/−), and their WT controls were used in this study. B1R−/− mice, which have been developed by one of the authors (M.B.), were obtained through the University of Arizona and have a genetic background of C57BL6 and 129/Sv. B2R−/− mice were derived from a breeding pair of homozygous knockout mice, generated by gene knockout mice. Male B1R knockout mice (B2R+/−) and their WT controls were used in this study. B2R−/− mice, which have been developed by one of the authors (M.B.), were obtained through the Jackson Laboratory (Bar Harbor, ME), and bred in our Animal Care and Use Facility at the Boston University Medical Campus. The strain is maintained on a mixed 129/SvC57BL6/J genetic background by matings of homozygotes. As controls, we used WT B6D2SvF2 mice, which were derived from mating a C57BL6/J male with a 129SJL/J female and were also obtained from the Jackson Laboratory. The animals, 8 to 12 weeks old and weighing 21 to 30 g, were housed in the animal quarters with a 12-hour light/dark cycle and were provided food (Purina Rodent Chow 5002) and distilled water ad libitum. All of the experiments were conducted in accordance with the guidelines for the care and use of animals approved by the Boston University Medical Center.

Coronary artery ligation was performed under anesthesia with IP pentobarbital sodium (50 mg/kg) and assisted ventilation. After recovery from surgery had no change in FS between days 0 and 21. All of the mice received ACE inhibition with lisinopril (ie, the WT mice, the B1R knockout groups, and 3 B2R knockout groups. One group from each of these groups survived to coronary ligation followed by ACE inhibition. In these last 3 groups, immediately after surgery, lisinopril was given in the drinking water at a concentration of 5 mg/kg per day for the next 3 weeks, until the end of the experiment. Before and at 3 weeks after the procedure, all of the animals had an indirect measurement of BP and evaluation of cardiac hemodynamics. They were then euthanized and their hearts were removed, weighed, and processed for analysis of gene expression of BRs, as well as genes of selected enzymes relevant to cardiac function and metabolism, ie, nitric oxide synthase 3 (NOS3), pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), and muscle carnitine palmitoyl transferase-1 (mCPT1).

Measurement of BP and Cardiac Function

Systolic BP was measured in conscious awake mice using a noninvasive computerized tail-cuff system (BP-2000, Visitech Systems), as described previously. This measurement has been ascertained in our laboratory to correlate closely with direct intraarterial measurement of BP. Fractional shortening (FS), an index of left ventricular systolic capacity, was measured on awake conscious mice at the beginning and end of the experiment, with a Doppler echocardiographic system (Acuson C256), as described previously. Data were obtained by 2 collaborating observers and analyzed by 1 of them in a blind fashion.

Assessment of Gene Expression By Quantitative Real-Time RT-PCR

The reverse transcription reagents (Applied Biosystems) were used to synthesize cDNA in a 50-μl reaction containing 1 μg of DNase I-treated (Ambion) total RNA. Reverse transcription reaction was carried out at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. The cDNA was analyzed immediately or stored at −20°C for later use. Quantitative real-time RT-PCRs were performed with the ABI Prism 7900HT Sequence Detection System using a TaqMan-based or SYBR green-based protocol (Applied Biosystems). We assessed the expression of the following genes: B1R, B2R, NOS3, PDK4, and mCPT1. We used the TaqMan gene expression assays from Applied Biosystems to assess the expression of the B1R gene (Mm00432059) and B2R gene (Mm00437788). The SYBR green–based protocol was used for the 3 enzymes and the control GAPDH gene with the following primers: NOS3 reverse: 5′-CTCTCTGCTCTCTCTCATT-3′; NOS3 forward: 5′-CAGCCTGCTAAAGTGACCATT-3′; PDK4 reverse: 5′-TACTCCAGGATGCCTTGAACC-3′; PDK4 forward: 5′-GGCCAGAGAACCA-GAAGCC-3′; mCPT1 reverse: 5′-GCTTACACACACAGTGCTCG-3′; mCPT1 forward: 5′-CCCTCAACACCGAACCTGTG-3′; GAPDH reverse: 5′-GGATGGGAGGAGGATGTTC-3′; and GAPDH forward: 5′-TCACACACCGAACCTGTG-3′. All of the reactions were run in triplicate and included negative controls. After the initial denaturation at 95°C for 10 minutes, the cDNA products were amplified for 40 cycles consisting of denaturation at 95°C for 15 seconds and annealing and extension performed in a single step at 60°C for 1 minute. The SDS 2.1 software generated standard curves from 10-fold serial cDNA dilutions, and the threshold cycle was normalized for each standard curve. The slopes were between −3.12 and −3.65, where −3.33 corresponds with 100% efficiency of the PCR. To assess the expression of the B1R and B2R genes, the copy numbers for all of the samples were normalized with the data obtained from 18S (TaqMan Ribosomal RNA control reagents, part 4308329, Applied Biosystems). The expression levels for 3 enzymes were normalized with GAPDH endogenous controls.

Statistical Analyses

Data are expressed as means±SEMs. Statistical comparisons were done by Student t test. P<0.05 was considered to indicate a significant difference.

Results

BP Measurements

In all 3 groups of animals that, after coronary artery ligation, received ACE inhibition with lisinopril (ie, the WT mouse, the B1R gene knockout mice, and the B2R gene knockout mice), there was a significant fall in BP after 3 weeks by 14.3±0.4 mm Hg, 8.7±0.6 mm Hg, and 24±1 mm Hg, respectively (P<0.05). A significant decrease in BP by 5.8±0.2 mm Hg was also observed in the B2R gene knockout mice that underwent sham operation. There were no significant changes in the other groups. All of the BP data are shown in the Table.

Cardiac Function Measurements

Evaluation of systolic function by the degree of FS is also shown in the Table. The 3 groups that underwent sham surgery had no change in FS between days 0 and 21. All of
Gene Expression Analysis

Expression of both BR genes and genes to the enzymes NOS3, PDK4, and mCPT1 was assessed in all of the groups of animals. The level of expression in the WT sham-operated mice is the normal rate of expression of each gene.

Figure 1A shows the BR gene expression in the groups submitted to various manipulations. In the WT groups, after coronary ligation with or without ACE inhibition, there was little change in BR gene expression. In the B1R−/− groups, the BR became highly overexpressed, even without further manipulation, as expected, and the coronary ligation with or without ACE inhibition produced small additional changes that were quantitatively and directionally similar to those seen in the WT groups with the same manipulations.

Figure 1B shows the BR gene expression in the various groups. Coronary ligation alone produced a small but significant change in the WT mice, but concurrent ACE inhibition caused an even greater downregulation of this gene. In the B1R−/− groups, there was a significant upregulation of the BR gene in the sham-operated mice as expected, but this overexpression was significantly less in the coronary ligation group and was further suppressed by concurrent ACE inhibition, indicating that both of these manipulations had a downregulating effect on the overexpressed BR. These changes were directionally similar to those of the WT mice but far more exaggerated.

Figure 2 shows the NOS3 gene expression in the various groups. There were small differences between the WT and BR knockout subgroups. However, the BR knockouts had a marked overexpression of this gene at baseline (sham operated), and, contrary to the other 2 strains, the coronary

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Cal indicates coronary artery ligation.

*P<0.05 at day 21 compared with day 0 within the group.

the groups submitted to coronary ligation had significant decreases in FS from their respective baselines. However, in the WT mice, which had a decrease of FS by 27±1% if untreated, treatment with lisinopril reversed this to only 8±0.3%. In the B1R−/− mice, which had a decrease in FS by 24±0.8% if untreated, there was essentially no difference if treated with lisinopril (a 28±0.5% decrease). On the contrary, the B1R−/− mice, which had a 32±1.5% decrease in FS after coronary ligation, exhibited a further significant deterioration in systolic function when treated with lisinopril (a 48±1.8% decrease). The difference between these responses (ie, an additional decrease in FS by 4% versus 16% after ACE inhibition) is significant at P<0.5.

Figure 2 shows the NOS3 gene expression in the various subgroups (WT, B1R gene knockouts, and B2R gene knockouts at baseline and after coronary ligation with or without concurrent ACE inhibition). The transcript copy numbers were normalized with the data obtained from the 18S control, and means±SEs were calculated. *P<0.05 between groups, #P<0.05 from the group’s baseline (ie, sham-operated subgroup). Cal indicates coronary artery ligation; Lis, lisinopril.
ligation with or without concurrent ACE inhibition clearly produced a partial suppression of the overexpressed gene.

Figure 3 shows the patterns of the PDK4 gene expression in the various subgroups. In the WT mice it remained unaffected by coronary ligation with or without ACE inhibition. In the BrR knockout mice it was significantly suppressed, but concurrent ACE inhibition seemed to restore it, whereas in the BrR knockouts, it was greatly overexpressed and became suppressed by the various manipulations.

Figure 4 shows the pattern of the mCPT1 gene expression. It was not significantly different in the 3 WT subgroups or in the 2 BrR knockouts at baseline. The coronary ligation produced a significant suppression of this gene in both BrR and B2R gene knockouts that tended to be restored by concurrent ACE inhibition only in the BrR knockouts.

Discussion
As would be expected from existing knowledge, these studies confirm that, at 3 weeks after a large acute MI caused by obstruction of the left anterior descending coronary artery, there is a significant decrease in left ventricular systolic capacity, as shown by a 27% reduction in FS; but if concurrent therapy with an ACE inhibitor is started immediately, the systolic capacity of the myocardium can be preserved to a large extent in the WT mice. This effect is partly attributed to inhibition of angiotensin II and partly to potentiation of bradykinin, believed to act via its B2R, because in previous reports, it could be partly reversed by B2R antagonists. In BrR mice, it was reported that cardiac remodeling and function post-MI was no different from that of WT mice, unless they were submitted to concurrent pharmacological BrR blockade, suggesting that the upregulation of the B2R was sufficient to compensate for lack of the BrR. However, in our BrR- mice, although the B2R gene was already significantly (by 5-fold) upregulated, as shown in the sham-operated subgroups, there was no improvement in left ventricular systolic capacity with ACE inhibition post-MI. Actually, this treatment was associated with a significant downregulation of the B2R in both the WT and BrR- mice, roughly to the same extent. The data would suggest that both BRs are needed for bradykinin to exert its full cardioprotective action. It is also consistent with previous findings that the BrR, when present, becomes activated with various manipulations and can assume vasoactive properties similar to those of the B2R and may, therefore, contribute to these hemodynamic findings. Lack of this contribution in the BrR- mice would have resulted in hampered ability of the ACE inhibitor to restore cardiac function, although at baseline the BrR had been upregulated.

More surprising, however, was the functional response of the BrR- animals; like the other 2 strains, if untreated, they had a significant decrease in FS at 3 weeks post-MI. It has been shown in the past that the cardioprotective capacity of ACE inhibition post-MI is diminished in the BrR knockout mice; but in the present experiments, when these mice were treated with concurrent ACE inhibition, not only did they not improve, they actually showed a greater deterioration, by 52%, in systolic capacity, compared with 39% in their untreated counterparts. The greater systolic BP lowering with ACE inhibition in this group evidently reflects the diminished systolic capacity of the infarcted myocardium. As shown in Figure 1A, the BrR was significantly overexpressed in these animals at baseline without other manipulation, as expected. Its expression increased further with coronary

Figure 2. NOS3 gene expression analysis by real-time PCR in the various subgroups. The transcript copy numbers were normalized with the data obtained from the GAPDH control, and means±SEs were calculated. *P<0.05 between groups, #P<0.05 from the group's baseline (ie, sham-operated subgroup). Cal indicates coronary artery ligation; Lis, lisinopril.

Figure 3. PDK4 gene expression analysis by real-time PCR in the various subgroups. The transcript copy numbers were normalized with the data obtained from the GAPDH control, and means±SEs were calculated. *P<0.05 between groups, #P<0.05 from the group's baseline (ie, sham-operated subgroup). Cal indicates coronary artery ligation; Lis, lisinopril.

Figure 4. mCPT1 gene expression analysis by real-time PCR in the various subgroups. The transcript copy numbers were normalized with the data obtained from the GAPDH control, and means±SEs were calculated. *P<0.05 between groups, #P<0.05 from the group's baseline (ie, sham-operated subgroup). Cal indicates coronary artery ligation; Lis, lisinopril.
ligation (unlike that of the B_2R that decreased in the B_2R^{-/-} mice post-MI) and was not significantly diminished by concurrent ACE inhibition. As mentioned earlier, in the absence of B_2R, the upregulated B_1R seem capable of taking over to a large extent the vasoactive properties of the B_2R, because they, too, can activate the arachidonic acid-NO cascade.\textsuperscript{24} Indeed, the NOS3 gene was significantly upregulated in both BR gene knockout groups, probably as a compensatory reaction to the loss of either BR, although this upregulation was much more pronounced in the B_2R knockout. However, the metabolic properties of bradykinin, ie, enhanced glucose transport and use, are a direct function of the B_2R\textsuperscript{10,11} and are lost in its absence.\textsuperscript{32,33} Therefore, in the B_1R^{-/-} animals, the compensatory upregulation of the B_2R would restore blood flow to the ischemic myocardium but would not be able to compensate for the diminished metabolic capacity of starving myocardial cells. The importance of improved glucose use in enhancing the viability of ischemic or injured myocardium is now well recognized, as shown by the recent reintroduction of the glucose-insulin-potassium treatment after acute MI or cardiac surgery.\textsuperscript{34} In an effort to explore the consequences of loss of each BR on myocardial metabolism, we chose to evaluate changes in expression of 2 enzymes, the PDK4, which is involved in carbohydrate metabolism,\textsuperscript{35} and the mCPT1, which has a crucial role in the regulation of fatty acid oxidation that sustains myocardial metabolism.\textsuperscript{36} In the WT mice, neither one showed significant change with coronary ligation with or without concurrent ACE inhibition. However, in the B_2R knockout mice with overexpressed B_1R, the PDK4 was significantly suppressed, whereas in the B_2R knockout, it was significantly upregulated, probably in compensatory reaction to loss of the contribution of B_2R to glucose transport and metabolism. Post-MI, it became greatly suppressed in the B_2R knockout, but ACE inhibition restored it toward normal in both BR knockout subgroups, in line with the known benefits of ACE inhibitors on glucose metabolism. The mCPT1 gene expression was variable but essentially unaffected in the WT subgroups of these post-MI survivors. However, it was suppressed by the coronary ligation in both BR gene knockout groups, and this suppression was restored by ACE inhibition only in the B_2R knockout. The suppression persisted in the B_2R knockout, which exhibited a significant additional deterioration in their cardiac function.

In fact, this significant additional deterioration of cardiac systolic capacity after ACE inhibition in the B_2R gene knockout mice was the only unexpected and intriguing finding of these experiments. Interpretation of this result can only be speculative at this time. It would appear that, having remained overexpressed, the B_1R may have rendered the injured myocardium more vulnerable to inflammatory mediators, which would, therefore, cause further injury, despite ACE inhibition. A previous study, using isolated perfused hearts submitted to coronary ischemia/reperfusion injury, found that hearts from B_2R knockout mice had sustained a significantly smaller infarct compared with hearts from WT mice, suggesting that the B_2R had a detrimental effect during myocardial ischemia.\textsuperscript{37} ACE inhibition, which blocks the inactivation of bradykinin, may enhance the conversion of the peptide from a B_2R to a B_1R agonist, thus contributing further to the adverse consequences of B_2R activation in the heart.

Activation of the B_1R occurs under all sorts of conditions causing tissue injury and inflammation.\textsuperscript{43} Indeed, a significant upregulation of B_1R mRNA has been reported at 6 and 24 hours after acute MI in rats.\textsuperscript{38} Although in our present studies, 3 weeks after MI, there was no longer a significant difference in B_1R gene expression in either WT or B_2R^{-/-} from their respective baselines, it is very likely that such alterations may have occurred in earlier stages and may have contributed to the subsequent functional alterations. A significant acute elevation of proinflammatory cytokines has been reported within hours post-MI,\textsuperscript{39} and even in stable coronary heart disease, there is significant elevation of a variety of markers of chronic inflammation,\textsuperscript{40,41} which may be capable of inducing B_1R gene expression. Another possibility is that the augmented B_1R activity enhances NO release via inducible NO synthase that can be noxious when converted to peroxynitrite. In short, in the case of the B_2R^{-/-} mice, the overexpressed B_1R may exert multiple effects, on the one hand promoting further tissue injury, whereas on the other, offering some degree of protection via its vasodilatory properties, yet being unable to compensate for the loss of metabolic properties that would enhance glucose use by the injured myocardium.

Some limitations of our study should be acknowledged. The extent of myocardial tissue damage was not directly assessed but only inferred from the degree of functional deterioration, which is believed to reflect the extent of anatomic injury, and the speculation regarding adverse metabolic consequences in knockout animals is based on gene expression studies rather than direct evaluation of ATP or glucose uptake by the myocardium. Nevertheless, these limitations do not diminish the value of our findings, namely, the fact that bradykinin, acting on an upgraded B_2R unopposed by a fully functional B_1R, exerts a nefarious effect on the myocardium.

Perspectives

In conclusion, these studies confirm the fact that minimization of myocardial tissue injury by ACE inhibition after acute MI is partly because of the cardioprotective properties of bradykinin, which have been repeatedly demonstrated in the past by either their reversal with specific bradykinin B_2R antagonists\textsuperscript{8,28} or their enhancement with gene treatment.\textsuperscript{42} By comparing WT with genetically engineered mice with deleted B_2R or B_1R, we have shown that, contrary to previous belief, both receptors contribute to this property, and both must be available and fully functional to obtain the benefits of ACE inhibition. Indeed, in mice missing the B_2R, ACE inhibition cannot restore left ventricular systolic capacity, although the B_1R is upregulated; but, more surprisingly, in mice missing the B_2R, ACE inhibition actually worsens cardiac function, suggesting that, with upregulation of the B_1R, the noxious proinflammatory effects of bradykinin potentiation seem to predominate over the beneficial vasodilatory effects mediated by this receptor, which lacks metabolic benefits.

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Disclosures

None.

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