Cardioprotective Effects of a Selective B₂ Receptor Agonist of Bradykinin Post-Acute Myocardial Infarct

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BACKGROUND
The cardioprotective benefits of bradykinin are attributable to activation of its B₂ receptor (B₂R)–mediated actions and abolished by B₂R antagonists. The current experiments evaluated the cardioprotective potential of a potent, long-acting B₂R-selective agonist peptide analogue of bradykinin, the compound NG291.

METHODS
We compared the extent of cardiac tissue damage and remodeling and expression pattern of selected genes in mice submitted to acute myocardial infarct (MI) and treated for 1 week with either NG291 [Hyp³,Thi⁵,NChg⁷,Thi⁸]-bradykinin or with saline delivered via osmotic minipump.

RESULTS
Active treatment resulted in better ejection fraction (EF) 69 ± 1% vs. 61 ± 3.1% (P = 0.01), (vs. 85 ± 1.3% in sham-operated controls), fractional shortening (FS) 38 ± 4% vs. 32 ± 8% (NS) (vs. 53 ± 1.2 in sham-operated controls), and fewer markers of myocyte apoptosis (TUNEL-positive nuclei 4.9 ± 1.1% vs. 9.7 ± 0.03%, P = 0.03). Systolic blood pressure (SBP) at end point was normal at 110 ± 4.2 in actively treated mice, but tended to be lower at 104 ± 4.7 mm Hg in saline controls with decreased cardiac systolic capacity. Expression patterns of selected genes to factors related to tissue injury, inflammation, and metabolism (i.e., the B₂R, B₁R, endothelial nitric oxide synthase (eNOS), TNF-α, cardiomyopathy-associated C (Cmya3), and pyruvate dehydrogenase kinase isoenzyme 4 (PDK4)) showed that acute MI induced significant upregulation of these genes, and active treatment prevented or attenuated this upregulation, whereas the B₁R agonist itself produced no difference in the myocardium of sham-operated mice.

CONCLUSIONS
Treatment with a selective B₂R agonist initiated at the time of induction of acute MI in mice had a beneficial effect on cardiac function, tissue remodeling, and inflammation-related tissue gene expression, which may explain its structural and functional benefits.

Keywords: blood pressure; bradykinin analogue; cardiac function; cardiac remodeling; hypertension; myocardial ischemia


The cardioprotective properties of angiotensin-converting enzyme (ACE) inhibition have long been established¹ and have been mostly attributed to one of its two major effects, i.e., the suppression of angiotensin II; however, the second one, i.e., prolongation and potentiation of bradykinin² may be just as important.

Most of the physiologically significant actions of bradykinin are exerted via activation of its constitutively present type 2 receptor (B₂R), whereas its type 1 receptor (B₁R) is believed to be minimally expressed under normal conditions, but inducible by lipopolysaccharides, bacterial toxins, and inflammatory mediators resulting from tissue injury.³–⁵ Indeed, a large number of experimental studies using either pharmacologic receptor blockers or genetically engineered animals with deletion of selected genes have demonstrated that the B₁R is responsible for the beneficial hemodynamic and metabolic effects of bradykinin, including vasodilation, improved perfusion of heart and kidneys, enhanced insulin sensitivity, and protection from ischemic myocardial damage.⁶–¹⁶ On the contrary, activation of the B₂R after acute myocardial tissue injury seems to produce mixed effects: although by some it was reported to be beneficial,¹⁷ many studies, including our own, have found it to be detrimental because animals with blocked or deleted B₂R tolerate better such injury.¹⁸,¹⁹ Actually, we found that in animals with upregulated B₁R after deletion of the B₂R, the normally cardioprotective ACE inhibition inflicts further myocardial damage.¹⁸

On the basis of this knowledge, it would be anticipated that selective activation of the B₂R should be a promising pharmacologic target for reduction of infarct size and preservation of myocardial tissues after an acute ischemic event. The purpose of the studies described herein was to evaluate the cardioprotective potential of a highly potent, selective, and long-acting B₂R agonist.²⁰ To this aim, we compared the extent of cardiac...
tissue damage and remodeling in mice submitted to an acute myocardial infarct (MI) induced by ligation of the anterior descending coronary artery and treated for a week with this agent or with normal saline.

METHODS

Animals and procedures. Male FVB mice aged 10–12 weeks were used in these experiments, conducted according to our Institutional Animal Care and Use Committee guidelines.

Coronary artery ligation (CAL) was performed under isoflurane anesthesia (4% induction, 2% maintenance), and assisted ventilation on mice placed on a heating table to prevent hypothermia. The heart was exposed via a lateral thoracotomy, and an 8-0 silk suture was placed around the left anterior descending coronary artery and ligated under a microscope. Then, the chest was closed, and the lungs were reinflated using positive end-expiratory pressure.21

Four groups were studied (8–12 mice per group): sham-operated mice; sham-operated mice treated with continuous infusion of the B2R agonist; mice submitted to MI, treated with saline; mice submitted to MI, treated with the B2R agonist. The compound chosen was NG219 [Hyp3, Thi5, NChg7, Thi8]-bradykinin, kindly provided by Fernand Gobeil from Sherbrooke University, Sherbrooke, Quebec, Canada, as it is the most potent and long-acting agent described in his studies.20 The biological activity (i.e., receptor affinity) of this compound and its resistance to intravascular proteolysis were evaluated with in vivo and in vitro experiments using naturally expressed as well as recombinant human B2R.20

Treatment was started immediately after CAL. Either NaCl 0.9% or the B2R agonist at 1 µg/kg/h, dissolved in saline, were continuously delivered for 7 days through an osmotic minipump (Alzet, Model 2002; Alza, Palo Alto, CA) subcutaneously implanted in the scapular area at the time of the CAL procedure, under anesthesia. Before and at 7 days after the procedure, all animals had an indirect measurement of blood pressure (BP) and evaluation of cardiac hemodynamics by echocardiography. The left hemithorax of the animal was shaved, and the ultrasound transmission gel was applied to the precordium. The RMV 707 scanhead (30 MHz) was used to acquire short-axis views of the left ventricle (LV) from which M-mode images were recorded at the level of the papillary muscles. Ventricular wall dimensions and chamber diameters in systole (s) and diastole (d) were measured offline using built-in calipers, and fractional shortening (FS) was calculated according to the formula FS = (LVED − LVES)/LVED × 100 where LVED is the LV end-diastolic dimension and LVES is the LV end-systolic dimension. The semilipsoid formula was used to calculate chamber volumes in systole and diastole (Vd and Vv, respectively). These values were subsequently used to calculate the ejection fraction (EF) according to the formula EF = ((Vd − Vv)/Vd) × 100. All measurements were performed in a blinded fashion.

Histology and myocardial apoptosis. Cardiac sections were stained with hematoxylin and Masson’s trichrome in order to determine the infarct size. Infarct area and the total LV area of the myocardium were traced manually and measured automatically by the computer (ImageJ). Infarct size, expressed as a percentage, was calculated by dividing the sum of infarct areas by the sum of LV areas and multiplying by 100.

Detection of apoptotic cells was carried out with the use of a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. The procedure was performed using an in situ cell death detection kit, POD (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The percentage of apoptotic nuclei per section was calculated by counting the total number of TUNEL-staining nuclei divided by the total number of nuclei in 8–10 randomly selected fields at ×40 magnification, on two to three heart sections from six different hearts in each MI group.

Assessment of gene expression by quantitative real-time RT-PCR. The reverse transcription (RT) reagents (Applied Biosystems, Foster City, CA) were used to synthesize cDNA in a 50 µl reaction containing 1 µg of DNase I–treated (Ambion, Austin, TX) total RNA. RT reaction was carried out at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. 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 SYBR Green–based protocol (Applied Biosystems). We assessed the expression of the following genes: B2R, B2R, eNOS, TNF-α, PDK4, mCPT1, Cmya3, and GAPDH as a control housekeeping...
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**Statistical analyses.** Data are summarized as means ± s.e.m.s. Statistical comparisons were done by Student’s t-test, and Bonferroni correction was used for multiple comparisons. Differences were considered statistically significant at P < 0.05 except when a Bonferroni correction was used, where significance was at P < 0.01.

**RESULTS**

**Body weight and heart weight**

At baseline, all groups had similar body weight and BPs, and there were no further changes in the sham-operated animals (Table 1). In both MI groups, mice showed a tendency to lower body weight and enlarged hearts resulting in higher heart weight/body weight ratio that was somewhat more pronounced in the saline-treated than the B2R agonist-treated group, but these differences did not reach statistical significance (Table 1).

**Homodynamic effects of treatment**

The sham-operated animals had no difference in SBP and LV systolic function before and after treatment.

In the saline-treated MI group, there was a fall in SBP from 119 ± 4.1 to 104 ± 4.7 mm Hg, whereas in the B2R agonist-treated MI mice, there was a lesser SBP reduction from 120 ± 3.4 to 110 ± 4.2 mm Hg. None of these differences reached statistical significance.

Both MI groups had significant postinfarct reduction in EF and FS (Table 1). However, this reduction was less profound in actively treated mice as they exhibited significantly better parameters of LV systolic function. Specifically, EF was reduced by 23.58 ± 12.58% in the control group vs. 12.75 ± 6.36% in the actively treated group (P = 0.01). The FS was reduced by 20.8 ± 10.8% in the control vs. 11.75 ± 7.2% in the active treatment group, but this difference did not reach statistical significance.

**Infarct size and cardiac apoptosis**

No statistically significant difference was observed in infarct size between control saline-treated and B2R agonist–treated MI groups (28.66 ± 16% vs. 22.68 ± 12.64%, respectively, P = NS).

Apoptotic cardiomyocytes were detected by TUNEL staining in six hearts of each group submitted to MI 1 week after CAL. The ratio of TUNEL-positive nuclei to total number of nuclei in the control group was significantly higher compared to the active treatment group (9.7 ± 0.03% vs. 4.9 ± 1.1%, P = 0.03), indicating a favorable effect of the B2R agonist.

**Gene expression profiling in CAL and sham-operated animals**

Gene expression of B1R, B2R, eNOS, TNF-α, PDK4, mCPT1, and Cmya3 was evaluated in all groups. Neither of the sham-operated groups revealed any significant change in expression level of any genes. Figure 1a shows B1R gene expressions in the four groups. MI induced a marked upregulation in B1R gene...
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expression reaching ~6.5-fold elevation in the saline-treated group. However, in the B2R agonist–treated group, there was a partial suppression of the overexpressed gene. Similarly, B2R gene expression showed significant upregulation post-MI in the saline-treated group (Figure 1b), but this upregulation seemed to be prevented by B2R agonist administration. TNF-α gene expression level showed a significant elevation post-MI in the saline-treated group, whereas in the B2R agonist–treated group, it remained similar to that of the sham-operated animals (Figure 2a). Likewise, the B2R agonist prevented a marked increase of eNOS gene expression following CAL (Figure 2b). Expression of the mCPT1 gene was similar in all groups (Figure 3a), whereas expression of the PDK4 gene was upregulated by the MI in the saline-treated mice, but in the B2R agonist–treated group, it was similar to that of the sham-operated groups (Figure 3b). Finally, the control saline-treated group after CAL had a significant increase in Cmya3 gene expression that was prevented by the B2R agonist treatment (Figure 4).

**DISCUSSION**

These experiments demonstrate that treatment with a selective B2R agonist of bradykinin after acute ischemic event offers significant protection of cardiac structure and function. Indeed, mice submitted to acute MI by ligation of the left anterior descending coronary artery had significantly lesser loss of systolic function as shown by their better EF and lesser apoptosis of cardiomyocytes when treated for 1 week by constant infusion of a long-acting B2R-selective bradykinin analogue compared to saline-treated mice. These effects were accompanied by prevention of the upregulation of a number of inflammation-related genes that was seen in the salinetreated mice.

It has been reported in the past that bradykinin is implicated in the prevention and repair of myocardial damage following MI. Its beneficial cardiac effects are observed after administration of exogenous kinins or by kallikrein overexpression in transgenic rats. However, the mechanism of these cardioprotective effects has not been fully elucidated. A number of studies in the literature have proposed distinctive roles for B1R and B2R after MI, suggesting that the B1R is proinflammatory, whereas the B2R protects against tissue injury. Although the presence of both BRs is needed for bradykinin to exert its full cardioprotective action, as we have shown in a previous study with B2R gene knockout mice, it seems that the activation of B2R is the one that plays the pivotal protective role in post-MI myocardial damage. Experimental studies have shown that the cardioprotective effect of ACE inhibition...
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mice submitted to MI, treated with saline; B2-MI, mice submitted to MI, treated with B2R agonist; SHAM, sham-operated mice; SHAM-B2, sham-operated mice, treated with B2R agonist. *P < 0.05 between C-MI and B2-MI; #P < 0.05 between C-MI and SHAM. B2R, B2 receptor.

Figure 3 | (a) mCTP1 and (b) pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) gene expression levels at end point in the four groups of mice. C-MI, control mice submitted to MI, treated with saline; B2-MI, mice submitted to MI, treated with B2R agonist; SHAM, sham-operated mice; SHAM-B2, sham-operated mice, treated with B2R agonist.

Figure 4 | Cmya3 gene expression levels at end point in the four groups of mice. C-MI, control mice submitted to MI, treated with saline; B2-MI, mice submitted to MI, treated with B2R agonist; SHAM, sham-operated mice; SHAM-B2, sham-operated mice, treated with B2R agonist.

in MI can be abolished by co-treatment with a selective B2R antagonist. In fact, we have previously found that ACE inhibition in B2R gene knockout mice not only fails to improve cardiac function, but actually has a detrimental effect, evidently due to bradykinin's action on the upregulated B2R, whose proinflammatory action is potentiated.

The B2R plays an important role in cardiac remodeling in a variety of cardiac disease models. Yin et al., using either administration of bradykinin or genetically altered animal models, have also suggested that the B2R, but not the B1R, protects against ischemia/reperfusion-induced cardiac dysfunction by inhibiting apoptosis and limiting myocardial collagen proliferation and ventricular remodeling. However, this is for the first time that pharmacologic treatment with a B2R-selective agonist initiated immediately after acute MI is shown to diminish the loss of myocardial tissue and systolic functional capacity.

The current studies show that a novel stable bradykinin analogue with high-affinity/high-potency and selectivity as agonist for the B2R continuously administered in mice postacute MI beneficially influenced the hemodynamic parameters, cell apoptosis, and gene expression profiling. Specifically, this treatment resulted in a less compromised LV systolic function as indicated by the significantly higher EF in the active treatment group. Consistent with this, a previous study in genetically kininogen-deficient rats reported increase in regional myocardial blood flow around the ischemic lesion, resulting in a significant reduction in the size of MI after 24-h treatment with a nonpeptide B2R agonist.

B2Rs are widely expressed in the cardiovascular system and their activation exerts a vasodilatory effect. The absence of significant BP reduction in the sham-operated mice may be attributable to counter-regulatory adjustments upon chronic drug delivery. Both MI groups had a tendency to decreased BP, but it would appear that a better preserved LV performance in the actively treated mice resulted in a maintenance of BP closer to normal.

We also investigated the expression profile of a number of genes related to tissue injury using as comparator sham-operated mice. We found no significant effect of our compound in sham-operated mice in either gene expression or parameters of hemodynamic function. Evidently, at this dose and administration, the B2R agonist itself does not significantly affect B1R or B2R gene expression levels.

In contrast, acute myocardial ischemia produced significant upregulation in a number of genes related to inflammation, perfusion, and metabolism, such as the B2R, B2R, TNF-α, and PDK4, as seen in the saline-treated mice. The active treatment prevented to a large extent these changes, as their pattern was similar or much closer to that of the sham-operated mice. In particular, expression of the B2R, which, as mentioned earlier, has repeatedly been reported to get rapidly activated post-MI and mediate the tissue damage, and the TNF-α, whose elevation accompanies heart failure, were virtually normalized in the actively treated mice. Likewise, upregulations of the B2R and eNOS genes may have been compensatory after acute ischemia, but were significantly lesser in the actively treated mice, suggesting that the ischemic stimulation was diminished. In fact, eNOS-derived nitric oxide has been reported to suppress myocardial O2 consumption in the postischemic period through modulation of mitochondrial metabolism and pathologically increased nitric oxide may be detrimental whereas, eNOS−/− mice exhibit reduced mitochondrial function compared to their wild-type counterparts.

Acute MI increased also significantly the expression of Cmya3 or Xirp2, a novel gene whose product is still unknown,
but is implicated in angiotensin-induced hypertensive/ischemic cardiomyopathy in mice.\textsuperscript{40,41} The B\textsubscript{2}R agonist prevented the upregulation of this gene in MI mice, whereas it had no effect of its own on this gene, as shown in the sham-operated mice. Preliminary data suggest that activation of this gene is an important mediator of cardiac tissue damage and remodeling.\textsuperscript{12}

The threefold increase in gene expression of the PDK4, which is related to glucose metabolism, would be consistent with the shift of myocardial metabolism to utilization of glucose under ischemic conditions. The B\textsubscript{2}R agonist prevented to a large extent this increase, although it is unclear whether this was due to facilitation of glucose metabolism by the B\textsubscript{2}R\textsuperscript{11–13} or whether it was the result of a lesser injury and better functional status of cardiomyocytes or both. Interestingly, the gene expression of mCPT1, which is involved in free fatty acid metabolism, that is normally the major source of energy for myocytes,\textsuperscript{13} was not affected by either acute MI or the B\textsubscript{2}R agonist treatment in these studies. Finally, the favorable effect of the B\textsubscript{2}R agonist on myocyte apoptosis is consistent with the findings reported in the literature.\textsuperscript{27,28,44}

Conclusions

The current experiments indicate that the combined anti-inflammatory and antiapoptotic effects of a potent and selective B\textsubscript{2}R agonist analogue of bradykinin administered after induction of acute myocardial ischemia can significantly attenuate the expected structural and functional cardiac damage. Although some earlier studies have suggested a possible contribution of the B\textsubscript{2}R to pathological processes, our results are consistent with a growing body of preclinical and clinical evidence indicating that B\textsubscript{2}R activation is beneficial in cardiovascular diseases.\textsuperscript{45}

Limitations

The small number of samples used for infarct size measurements and the short-term administration of the drug may have obscured further statistically significant differences between experimental groups. The use of TUNEL stain, as any other diagnostic assay for apoptosis alone, has limitations regarding its sensitivity and specificity. Complementary detection methods would be beneficial to study the complex process of apoptosis in depth.

Finally, we did not perform direct evaluation of glucose and free fatty acid metabolism in myocardial tissues. Such studies would further corroborate our findings, but their absence does not diminish the validity of our results.

Future perspectives

A longer administration or a higher dosage of this selective B\textsubscript{2}R agonist would be worth investigating. However, because the B\textsubscript{2}R exhibit rapid internalization and desensitization after chronic bradykinin stimulation,\textsuperscript{46} the myocardial effects of this treatment could be influenced by the downregulation of B\textsubscript{2}R. Clearly, further validation of these promising initial findings is necessary.

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