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Bradykinin inhibits development of myocardial infarction through B_2 receptor signalling by increment of regional blood flow around the ischaemic lesions in rats

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1 To identify the roles of endogenous kinins in prevention of myocardial infarction (MI), we performed the permanent ligation of coronary artery in rats.

2 The size of MI 12, 24, and 48 h after coronary ligation in kininogen-deficient Brown Norway Katholiek (BN-Ka) rats was significantly larger ($49.7 \pm 0.2\%$, $49.6 \pm 2\%$, and $51.1 \pm 1\%$, respectively) than that of kinin-replete Brown Norway Kitasato (BN-Ki) rats ($42 \pm 2\%$, $38.5 \pm 4\%$, and $41.5 \pm 1\%$).

3 Hoe140, a bradykinin (BK) B_2 receptor antagonist injected (1.0 mg kg^{-1}, i.v.) half an hour before, and every 8 h after, coronary ligation, significantly increased the size of MI in Sprague-Dawley rats. Aprotinin, a kallikrein inhibitor, which was infused intravenously (10,000 Units kg^{-1} h^{-1}) with an osmotic mini-pump, significantly increased the size of an MI 24 h after ligation.

4 When evaluated using microspheres, the regional myocardial blood flow around the necrotic lesion in BN-Ka rats 6 h after ligation was reduced more than that in BN-Ki rats with MI by 41–46%. The same was true in Hoe140-treated BN-Ki rats.

5 FR190997, a nonpeptide B_2 agonist, which was infused (10 μg kg^{-1} h^{-1}) into the vena cava of BN-Ka rats for 24 h with an osmotic mini-pump, caused significant reduction in the size of MI ($38 \pm 3\%$), in comparison with the size in vehicle solution-treated rats ($51 \pm 3\%$). The size of MI in FR190997-treated BN-Ka rats was the same as in BN-Ki rats.

6 These results suggested that endogenous kinin has the capacity to reduce the size of MI *via* B_2 receptor signalling because of the increase in regional myocardial blood flow around the ischaemic lesion.

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Abbreviations: ACE, angiotensin converting enzyme; BK, bradykinin; BN-Ka, Brown Norway Katholiek; BN-Ki, Brown Norway Kitasato; LV, left ventricle; MI, myocardial infarction; MBP, mean blood pressure; SD, Sprague-Dawley; TTC, triphenyltetrazolium chloride

Introduction

It has been reported that angiotensin converting enzyme (ACE) inhibitors have many beneficial effects in cardiovascular disease (Linz *et al*., 1995). Occlusion of the coronary artery causes cardiomyocyte dysfunction and eventual tissue necrosis. ACE inhibitors are known to protect myocardium against ischaemia and to prevent ischaemia reperfusion injury (Fleetwood *et al*., 1991; Linz *et al*., 1986; Van Gilst *et al*., 1987). In particular, ACE inhibitors reduced the extent of cellular necrosis of the myocardium after coronary ligation (Ertl *et al*., 1982; Hock *et al*., 1985). ACE is the same enzyme as kininase II, one of the major kinin-degrading enzymes (Linz *et al*., 1995), so ACE inhibitors may accumulate bradykinin in the circulation or in the tissues where kinins were generated. Some beneficial effects of ACE inhibitors were reported to be cancelled by a kinin antagonist (Hartman *et al*., 1993).

The size of the myocardial infarctions (MI) of animals that had received a continuous infusion of kinin into the coronary artery before the coronary occlusion was reported to be reduced significantly (Martorana *et al*., 1990). Further, it was recently reported that delivery of the gene of tissue kallikrein, a kinin-generating enzyme, attenuated MI and reduced apoptosis, although delivery of kallikrein gene was performed 1 week before the induction of MI (Yoshida *et al*., 2000). Accordingly, it is probable that endogenous bradykinin may play an important role in the cardioprotective effect against MI, and that ACE inhibitors reduced MI *via* the accumulation of endogenous kinins. However, the mechanism of the protective effects of kinins against MI has not been fully elucidated.

Kinins are well known to dilate the resistance vessels and to increase the coronary blood flow (Linz *et al*., 1995). Thus, it is plausible that the endogenous kinins may inhibit the necrosis of the myocardium by increment of blood supply

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through this vasodilation. In the present experiment, we used kininogen-deficient mutant rats (Hayashi *et al*., 1993; Majima & Katori, 1995), which are incapable of generating kinin, to evaluate the contribution of endogenous kinins to the prevention of MI, and administered FR190997, a nonpeptide mimic of bradykinin, which is highly resistant to kinin-degrading enzymes and stimulates B_2 receptors (Aramori *et al*., 1997; Majima *et al*., 2000). This study elucidates the mechanism of action of endogenous kinins in reducing the severity of MI by improving the regional blood flow in the myocardium.

Methods

Animals

Brown Norway Katholiek (BN-Ka) rats (*Rattus norvegicus*, BN/fMai) were initially obtained from the Katholieke Universiteit of Leuven, Belgium. Normal rats of the same strain, Brown Norway Kitasato (BN-Ki) rats were transferred from the Microbiological Association, Frederick, MD, U.S.A., and were kept at Kitasato University. All rats used were males, in most cases 10–12 weeks old, but in some experiments, Sprague-Dawley (SD) strain rats 6 weeks old (SLC, Hamamatsu, Japan) were used. The body weight of these animals was approximately 170–200 g. The size of these animals were adequate to produce the MI model (Sasaki *et al*., 1988; Kuribayashi *et al*., 1996).

The animals were housed at a constant humidity ($60 \pm 5\%$) and temperature ($25 \pm 1^{\circ}C$), and kept on a 12-h light/12-h dark cycle throughout the experiments. They were given normal rat chow (NMF, Oriental Yeast Corp., Tokyo, Japan). The number of animals (n) used for each experiment is stated in the corresponding section. The study was performed in accordance with the guidelines for animal experiments of Kitasato University School of Medicine.

Basal haemodynamics of BN-Ka rats was not different from those of normal BN-Ki rats, if they were fed with low sodium diet and tap water without high sodium (Majima & Katori, 1995). Furthermore, under such a condition, the weights of major organs in BN-Ka rats including hearts were not also different from those in BN-Ki rats.

Induction of myocardial infarction

MI was induced according to the method of our previous report (Kuribayashi *et al*., 1996). Briefly, rats were anaesthetized lightly with ether, and the thorax was opened. The left main coronary artery was ligated 1–2 mm below its origin with white silk suture thread (4-0, Natsume Ind., Tokyo). The heart was then replaced in the thorax, which was immediately closed.

Administration of drugs

A bradykinin antagonist, Hoe140 (1.0 mg kg^{-1}, dissolved in physiological saline, Peptide Institute) (Wirth *et al*., 1991a) was injected intravenously under light ether anaesthesia half an hour before, and every 8 h after, coronary ligation. A B_1 receptor antagonist, des Arg^9-D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8] bradykinin (des Arg^{10}-[Hoe140]) (Wirth *et al*., 1991b) which was obtained from Peninsula Laboratories (San Carlos, CA, U.S.A.), was also administered (1.0 mg kg^{-1}) in the same manner as Hoe140. This dose of Hoe140 was quite sufficient to block B_2 receptor *in vivo* (Wirth *et al*., 1991a). In a separate experiment, a low dose of Hoe140 (0.3 mg kg^{-1}) was also tested. A nonpeptide mimic of bradykinin, FR190997 (8-[2,6-dichloro-3-[N-[(E)-4-(N-methylcarbamoyl) cinnamidoacetyl]-N-methyl- amino]benzyloxy]-2-methyl-4-(2-pyridylmethoxy)-quinoline) (Aramori *et al*., 1997) (10 μg kg^{-1} h^{-1}, suspended in 0.01 N HCl at a concentration of 2.5 mg ml; and itself a gift from Fujisawa Pharmaceutical Corp., Osaka, Japan), was administered immediately after coronary ligation for 24 h by continuous infusion into the vena cava (8 μl h^{-1}) using a microosmotic pump (Alzet model 2001D, Alza Corp., Palo Alto, CA, U.S.A.), which was primed according to the protocol from Alza Corp., and was implanted into the subcutaneous tissue of the backs of the rats. The tip of the cannula to infuse FR190997 was placed in the vena cava of each animal. This compound was first dissolved in 0.1 N HCl, and then diluted 1 : 10 with physiological saline. Vehicle solution (saline containing HCl) was administered to control animals using the same types of pump and cannula, in order to evaluate the specific activity of FR190997. Aprotinin (10,000 unit kg^{-1} h^{-1}, dissolved in 0.9% NaCl/H_2O, Wako Pharmaceutical Corp., Osaka, Japan) (Majima *et al*., 1991) was administered from 30 min after coronary ligation for 24 h by continuous intravenous infusion to the cervical vein using a microosmotic pump (Alzet model 2001D) implanted subcutaneously in the back. Trypsin inhibitor from soybeans (SBTI) (0.4 mg kg^{-1} h^{-1}, dissolved in 0.9% NaCl in H_2O, Wako Pharmaceutical Corp., Osaka, Japan) (Majima *et al*., 1993) was also administered from 30 min after coronary ligation for 24 h by continuous intravenous infusion using a microosmotic pump (Alzet model 2001D).

Evaluation of infarction size

The infarction size of the hearts at 6, 12, 24 and 48 h after ligation were evaluated by the previously reported methods (Sasaki *et al*., 1988; Kuribayashi *et al*., 1996). The heart was rapidly excised and sliced into four transverse rings (rings A, B, C and D, starting from the base) at 2–2.5 mm intervals. Three transverse rings (rings B, C and D) of the hearts were incubated in a 1% triphenyltetrazolium chloride (TTC) solution in a 0.09 M phosphate buffer (pH 8.5; 37°C, 20 min). TTC is reduced to a red formazan by electron acceptance. This reaction does not occur in the areas in which the mitochondria or oxidative systems have been damaged or impaired. Thus, the lack of staining demarcated the injured areas from the intact areas. The stained and unstained areas of the basal surface of each ring were photographed with a digital camera, NV-DCF1 (Matsushita Electric Industrial Co., Ltd. Osaka, Japan), and their sizes were measured using NIH computer image software. The unstained areas were taken to be the infarct areas and were expressed as a percentage of the total cut area of the left ventricle (%LV).

Measurement of blood pressure

Mean arterial blood pressure (MBP) was determined for 1 h in conscious and unrestrained rats, as reported previously

(Majima *et al*., 1993), 23 h after administration of the Hoe140 or FR190997. The rats were anaesthetized with light ether anaesthesia, and a polyethylene cannula (PE-10, Clay Adams, Parsippany, NJ, U.S.A.) was inserted into the abdominal aorta through the femoral artery 2 days before the experiments. The cannula was connected to a PE-50 cannula (Clay Adams, Parsippany, NJ, U.S.A.) and exteriorized in the interscapular region. A blood pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan) was attached to the other end of the intra-arterial cannula, and the mean arterial blood pressure was monitored on a polygraph (WS-641-G, Nihon Kohden, Tokyo, Japan) (Majima *et al*., 1993). Starting 30 min after the connection of the transducer, recordings were made for over 1 h in the rats, which were kept in separate cages.

Determination of a stable bradykinin metabolite, BK-(1-5), in the blood and in the myocardium

Six hours after the induction of MI, 5 ml of blood was collected from the carotid artery under light ether anaesthesia through a cannula into plastic tubes containing 20 ml of ice-cold absolute ethanol. After shaking, the mixture was centrifuged for 30 min at $3500 \times g$ at 0°C. The ethanol extracts (supernatants) were evaporated to dryness and were washed with diethylether three times to remove the lipids. The washed samples were dissolved in 4 ml of distilled water that had been acidified with 0.2 ml of 0.01 N HCl and were applied to a Sep-Pak C_{18} cartridge column (Waters Associates, Milford, MA, U.S.A.). After the samples had been washed with 12 ml of distilled water and 4 ml of 0.1 M acetic acid, BK and products of its degradation were eluted with 6 ml of 80% (v v^{-1}) acetonitrile containing 0.1 M acetic acid (Kauker *et al*., 1984). The kinin fraction was dissolved in 800 μl of the assay buffer, and the levels of BK-(1-5) were determined with an enzyme-linked immunosorbent assay (ELISA) kit (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) that was developed by us (Majima *et al*., 1996). The levels of BK-(1-5) were measured in rats with MI that underwent sham thoracotomy operations only. To determine the heart tissue levels of BK and BK-(1-5), the heart was excised, and was put into the liquid nitrogen. Then, the frozen heart was pulverized within a stainless cylinder cooled with liquid nitrogen. The powder of the heart was immediately put into 5 ml of 80% ethanol without thawing and mixed well with homogenizer. After centrifugation at $1500 \times g$ for 30 min at 4°C, the supernatant was dried with reduced pressure. The residue was washed with ether and the levels of BK and BK-(1-5) were determined as mentioned above with ELISA.

Measurement of regional myocardial blood flow with colored microspheres

Six hours after the left main coronary artery was ligated, measurement of the regional myocardial blood flow was done with colored microspheres. Regional myocardial blood flow was determined with a technique (Kowallik *et al*., 1991) using 15 μm microspheres (Dye-trak, Triton Technologies Inc.). A polyethylene cannula (PE-10, Clay Adams, Parsippany, NJ, U.S.A.) was inserted into the carotid artery under light ether anesthesia and the other end of it was connected to a PE-50 cannula (Clay Adams, Parsippany, NJ, U.S.A.). A blood pressure transducer (TP-200T, Nihon Kohden, Tokyo, Japan) was attached to the other end of the latter cannula, and the arterial blood pressure waves were monitored on a polygraph (WS-641-G, Nihon Kohden, Tokyo, Japan) (Majima *et al*., 1993). During the monitoring of the pressure, the polyethylene cannula was inserted until a change from an arterial blood pressure wave to a left ventricle pressure wave occurred. For each measurement, approximately 3×10^5 microspheres suspended in 0.3 ml of saline (containing 0.02% Tween 80) were injected into the left ventricle through the polyethylene cannula. Microsphere injection was followed by flushing with 0.2 ml of saline. After microsphere injection, the rats were exsanguinated and their hearts were excised under ether anaesthesia. The atrium and right ventricle were removed. The heart was rapidly excised and sliced into four transverse rings (rings A, B, C and D, starting from the base) at 2–2.5 mm intervals. One transverse ring (ring B) of each heart was stained with TTC. TTC staining is reduced to a red formazan by electron acceptance. This reaction is lost in the areas in which mitochondria or oxidative systems have been damaged. Ring B was cut into quadrants, starting from the centre of the TTC-unstained area (indicated as clear area) as quadrant a and working clockwise (quadrants b, c and d) (Figure 5). One ml of a 4 M KOH solution containing 2% Tween 80 was added to each quadrant of ring B to digest the tissue. The plastic tubes were closed, and allowed to stand for 48 h at 25°C. The digest was removed in plastic tubes until only 50 μl of the sample remained. Five μl of that solution was placed in a counting chamber (improved Neubauer type), and the number of microspheres was counted under a microscope at a magnification of $\times 100$.

Statistical analysis

Values were expressed as means $\pm$ s.e.mean. Factorial ANOVA and repeated measure ANOVA with the *post-hoc* test were used to evaluate the significance of differences. For comparison between two groups, Student's *t*-test was used. A P value less than 0.05 was considered to be significant.

Results

Comparison of sizes of myocardial infarctions in kininogen-deficient Brown Norway Katholiek rats and normal Brown Norway Kitasato rats

The time course of MI size outlined by TTC (triphenyltetrazolium chloride) staining in normal BN-Ki rats is shown in Figure 1 (squares). Ligation of the coronary artery increased the unstained area, which reached a plateau at 12 h. The MI size 12, 24, and 48 h after coronary ligation in BN-Ki was $42 \pm 2\%$, $38.5 \pm 4\%$, and $41.5 \pm 1\%$, of left ventricle (LV) size, respectively (Figure 1, squares). The final size of MI in kininogen-deficient BN-Ka rats (Figure 1, triangles) was reached within 12 h, and was significantly ($P < 0.05$) larger ($49.7 \pm 0.2\%$, $49.6 \pm 2\%$, and $51.1 \pm 1\%$ at 12, 24, and 48 h after ligation, respectively) than that observed in normal BN-Ki rats. One hour before the heart was excised at 24 h, MBP in BN-Ka rats was 99 ± 3 mmHg ($n = 4$), which was not statistically different from that determined in BN-Ki rats

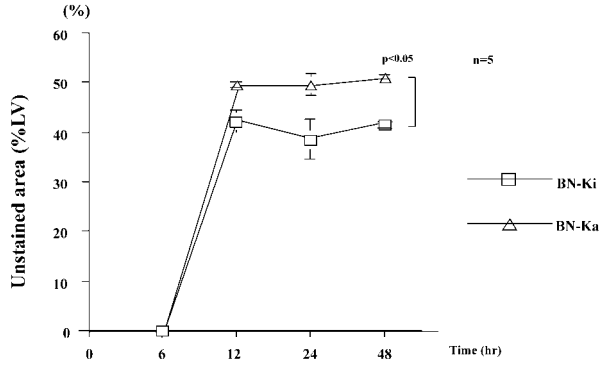

Figure 1 Time course of development of myocardial infarction in Brown Norway Katholiek rats and Brown Norway Kitasato rats. Myocardial infarction after coronary ligation was assessed using triphenyltetrazolium chloride (TTC) staining and expressed as a percentage of the unstained area of the left ventricle. Values are means ± s.e.mean obtained from five rats. ANOVA was used to evaluate the significance of differences. LV, left ventricle; BN-Ka, Brown Norway Katholiek rats; BN-Ki, Brown Norway Kitasato rats; hr, hour(s).

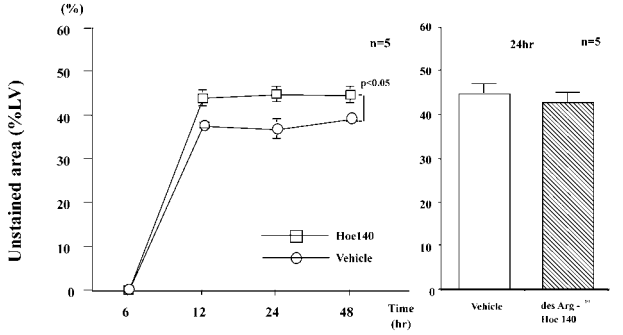

Figure 2 Effect of Hoe140, a B_2 receptor antagonist, and des Arg^{10}-[Hoe140], a B_1 receptor antagonist, on the development of myocardial infarction in SD rats. Myocardial infarction after coronary ligation was assessed by triphenyltetrazolium chloride (TTC) staining and expressed as percentage of unstained area of the left ventricle. Values are means ± s.e.mean obtained from five rats. ANOVA was used to evaluate the significance of differences. LV, left ventricle; Hoe140, SD rats receiving Hoe140; Vehicle, SD rats receiving vehicle; hr, hours.

(101 ± 3 mmHg, $n = 4$). The mortality of BN-Ka rats (three rats dead among 12 rats) was not significantly different from that in BN-Ki rats (four rats dead among 13 rats).

Effect of Hoe140 on myocardial infarction size

Hoe140, a bradykinin (BK) B_2 receptor antagonist, was tested in SD rats (Figure 2). When Hoe140 was injected (1 mg kg^{-1}) half an hour before, and every 8 h after coronary ligation, it increased the infarct size to 44 ± 3%, 45 ± 2%, and 44 ± 3% ($n = 5$) at 12, 24, and 48 h after coronary ligation. This value was statistically significant ($P < 0.05$) compared with that of vehicle-treated rats (Figure 2, right panel). However, des Arg^{10}-[Hoe140], a bradykinin (BK) B_1 receptor antagonist, did not affect the infarct size at 24 h (Figure 2, left panel).

When a low dose of Hoe140 was used, the infracted size 24 h after ligation was significantly increased to 43 ± 2% ($n = 5$), which was statistically larger than that in vehicle-treated rats.

One hour before the heart was excised at 24 h, MBP in Hoe140-treated rats was 102 ± 2 mmHg ($n = 5$), which was not statistically different from that determined in vehicle-treated rats.

Levels of BK-(1-5) in the circulation after myocardial infarction

The levels of BK-(1-5) in the blood collected from the carotid artery of SD rats with MI was determined by a specific ELISA 6 h after coronary ligation when the size of infarction was established (Figure 3). The levels of BK-(1-5) in rats with MI (183 ± 32 pg ml^{-1}) were increased significantly ($P < 0.05$) compared with those in sham-operated rats (105 ± 12 pg ml^{-1}).

The tissue levels of both BK and BK-(1-5), determined in the excised hearts were less than the detection limit (90 pg $heart^{-1}$) in sham-operated rats and rats with coronary ligation.

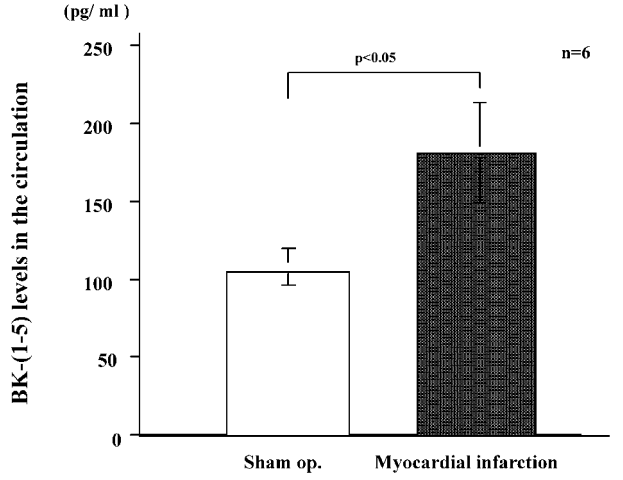

Figure 3 Kinin metabolite levels in the circulation. Blood was collected from the carotid artery 6 h after ligation, and BK-(1-5) levels were determined by a specific enzyme-linked immunosorbent assay. Values are means ± s.e.mean obtained from six rats. The *t*-test was used to evaluate the significance of differences. Sham op., samples from sham-operated rats.

Effect of kallikrein inhibitors on myocardial infarction size

Aprotinin, a tissue and plasma kallikrein inhibitor, which was continuously infused with an osmotic mini-pump into the jugular vein of SD rats (50,000 Units kg^{-1} 24 h^{-1}) starting immediately after coronary ligation, significantly ($P < 0.05$) increased the size of MI 24 h after ligation to 47 ± 3%LV compared with that observed in vehicle-treated rats (Figure 4).

SBTI, a plasma kallikrein inhibitor, which was continuously infused with an osmotic mini-pump into a cervical vein (30,000 Units kg^{-1} 24 h^{-1}) starting immediately after coronary ligation, also significantly ($P < 0.05$) increased the size of MI 24 h after ligation to 43 ± 3%LV (Figure 4). However, the magnitude of the effect of SBTI was less than that of aprotinin.

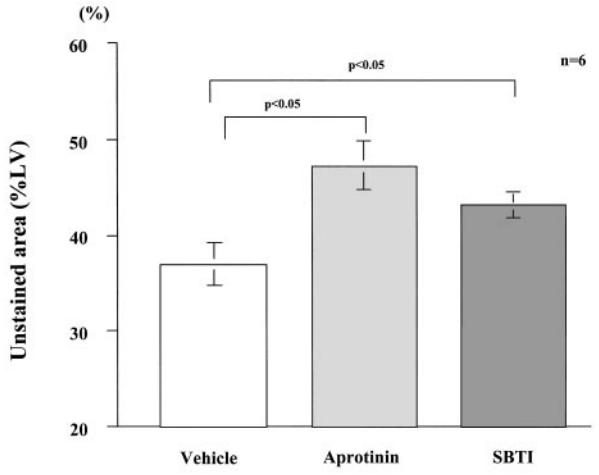

Figure 4 Effects of aprotinin and soybean trypsin inhibitor on the development of myocardial infarction in SD rats. Myocardial infarction after coronary ligation was assessed using triphenyltetrazolium chloride (TTC) staining and was expressed as percentage of unstained area of the left ventricle 24 h after coronary ligation. Values are means ± s.e.mean obtained from six rats. ANOVA was used to evaluate the significance of differences. LV, left ventricle; aprotinin, SD rats receiving aprotinin; SBTI, SD rats receiving soybean trypsin inhibitor; Vehicle, SD rats receiving vehicle solution.

Measurement of regional myocardial blood flow with microspheres in myocardial infarction of Brown Norway Katholiek rats and Brown Norway Kitasato rats

The numbers of microspheres in the left ventricle in quadrants a, b, c, and d were approximately 5000 pieces per g of heart weight in BN-Ki sham-operated rats (Figure 5). The numbers of microspheres in quadrants a, b, and d in BN-Ki rats 12 h after coronary occlusion were markedly reduced compared with those in sham-operated rats (Figure 5). There was no marked reduction in the numbers of microspheres in the opposite non-necrotic area (quadrant c) (Figure 5).

The numbers of microspheres in quadrants a, b, c, and d were reduced in BN-Ka rats with coronary ligation (Figure 5). The numbers of microspheres in the myocardium of BN-Ka rats with MI in quadrants b, c, and d, but not in quadrant a, were significantly reduced by 41%, 44%, and 46%, respectively, compared with those in BN-Ki rats with MI (Figure 5).

Measurement of regional myocardial blood flow with microspheres in myocardial infarction of Brown Norway Kitasato rats treated with Hoe140

Hoe140 (1.0 mg kg^{-1}, dissolved in physiological saline), a bradykinin B_2 receptor antagonist, injected half an hour before coronary ligation, significantly reduced the numbers of microspheres in sites b, c, and d (Figure 6), but no effect was seen on the number of microspheres in the myocardium in quadrant a, which bore a necrotic lesion (Figure 6).

Effect of FR 190997 on myocardial infarction size

FR190997, a nonpeptide mimic of bradykinin, which was infused (10 μg kg^{-1} h^{-1}) continuously into the vena cava in BN-Ka rats for 24 h with an osmotic mini-pump, caused significant ($P<0.05$) reduction in the size of MI (38 ± 3%LV), in comparison with the size in vehicle-treated rats (51 ± 3%LV) (Figure 7). The size of MI in FR190997-treated BN-Ka rats was the same as that in normal BN-Ki rats (Figure 7). One hour before the heart was excised, the MBP in FR190997-treated BN-Ka rats was 89 ± 1 mmHg ($n=7$), which was not significantly different from that in vehicle-treated BN-Ka rats.

Discussion

Kinins are potent vasoactive proinflammatory peptides that are released from their precursor kininogens by kallikrein (Bhoola *et al*., 1992). The BN-Ka used in the present study exists only as an experimental animal, which is incapable of kinin generation. Plasma kininogens are secreted in the liver. The plasma levels of both high-molecular weight kininogen and low molecular weight kininogen were very low. This was attributable to the lack of the ability in this strain to secrete these kininogen moieties from the liver. One-point mutation of alanine163 to threonine in the kininogen moiety is responsible to this lack of secretion, although the hepatic cells of the mutant BN-Ka synthesized kininogens that do not differ in molecular weight (about 110 kDa) when assessed by electrophoresis (Hayashi *et al*., 1993). Comparison of BN-Ka with normal BN-Ki allowed us to study the pathophysiological roles of endogenous kinins in many situations (Majima & Katori, 1995). In the present study, we clarified the protective action of endogenous kinins in a model in which MI was induced by coronary occlusion, by making use of these mutant rats and their normal counterparts. The development of MI in BN-Ka was certainly enhanced throughout the experimental period, indicating that endogenous kinin has a protective action in this model. The size of the MI reached a plateau 12 h after coronary occlusion. Thus, the endogenous kinin generated from kininogens effectively prevented the development of MI for up to 12 h after the onset of myocardial ischaemia.

The endogenous kinin levels in the circulation 6 h after ligation, which was the developing stage of MI, was certainly elevated during the induction of MI, judging from the elevated levels in a stable metabolite of kinins, BK-(1-5). Kinin generation occurred independently in the two kinin systems in the body: the plasma kallikrein-high molecular weight kininogen system and the tissue kallikrein-low molecular weight kininogen system. It was believed that during ischaemia, plasma kallikrein was activated through the activation of factor XII (Pitt *et al*., 1969). The reduction in pH in the tissue by hypoxia was reported to activate this system. In fact, the elevation of kinin levels in the blood returning from the coronary circulation of the heart after an infarction was previously reported (Hashimoto *et al*., 1977). Previous investigation of the source of kinin has been poor. Tissue kallikrein together with plasma kallikrein were inhibited by aprotinin, although this inhibitor can inhibit other enzymes than kallikrein such as plasmin, whereas SBTI inhibited plasma kallikrein strongly. The effect of aprotinin was more pronounced in increasing the MI size than that of SBTI, and the possible involvement of tissue kallikrein in kinin formation was not ruled out completely in the present

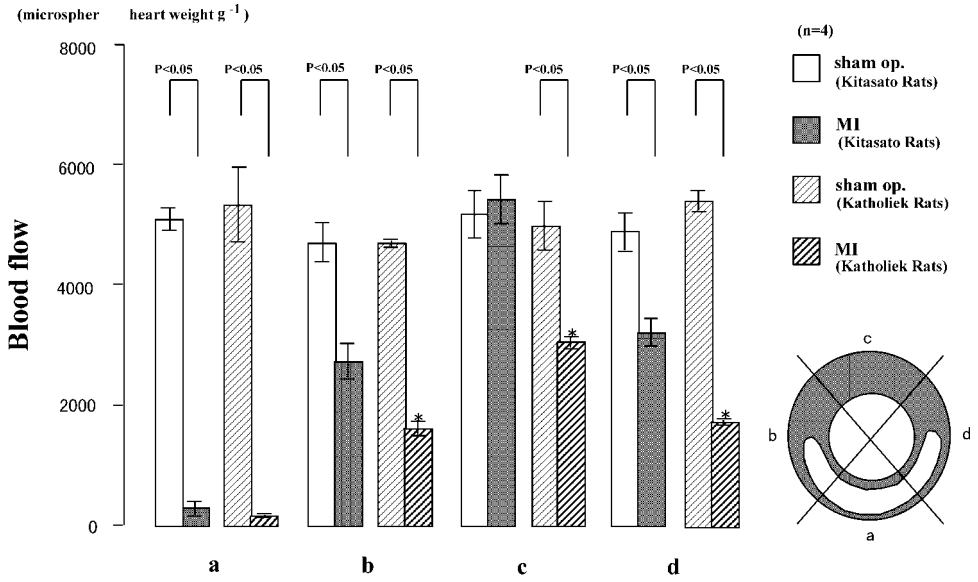

Figure 5 Measurement of regional myocardial blood flow with microspheres in kininogen-deficient Brown Norway Katholiek rats and normal Brown Norway Kitasato rats. Six hours after microsphere injection, the heart was excised and sliced into four transverse rings. One transverse ring (ring B, see the text) of the heart was stained by TTC. Ring B was cut into quadrants, starting from the center of the TTC-unstained area (indicated as clear area) as quadrant a and working clockwise (quadrants b, c and d). The number of microspheres in quadrants a, b, c, and d was counted under a microscope. Values are means ± s.e.mean obtained from four rats. ANOVA was used to evaluate the significance of differences. *Compared with results from BN-Ki rats with coronary ligation.

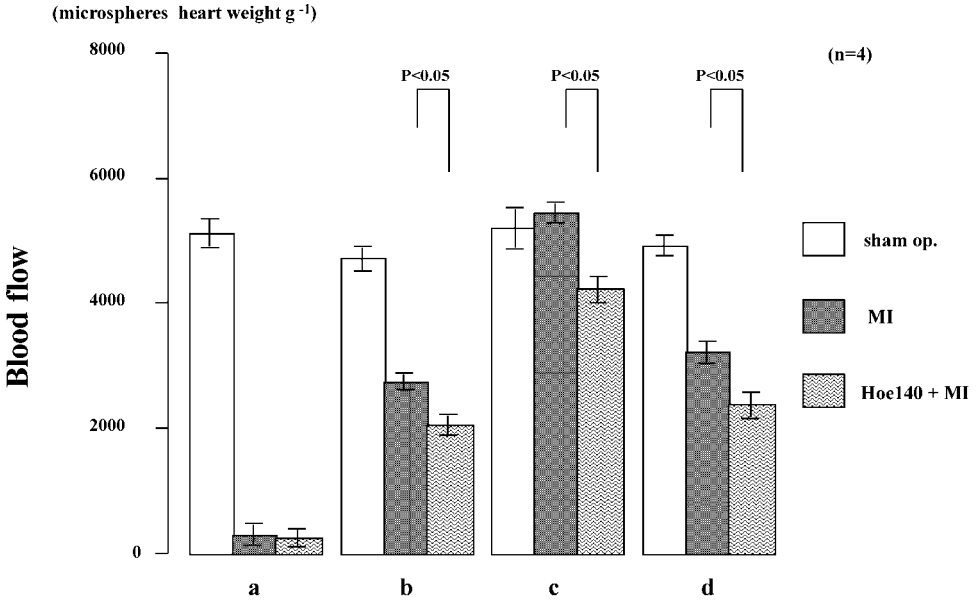

Figure 6 Effect of Hoe140 on regional myocardial blood flow in normal Brown Norway Kitasato rats. Regional blood flow was determined by the same method as that used in Figure 5. Values are means ± s.e.mean obtained from four rats. ANOVA was used to evaluate the significance of differences. Sham op., rats that had sham operation; MI, rats with coronary ligation; Hoe140 + MI, Hoe140-treated rats with coronary ligation.

study. Kinins generated from the plasma kallikrein-high molecular weight kininogen system were reported to exert a protective effect on myocardial injury (Yang *et al*., 1997). However, another kinin generating system, the tissue kallikrein-low molecular weight kininogen system, may be responsible for these protective effects, since tissue kallikrein gene delivery attenuated MI together with apoptosis in rat coronary occlusion models (Yoshida *et al*., 2000). Expression of tissue kallikrein in the blood vessels and myocardium has frequently been reported (Nolly *et al*., 1993; 1994). Thus, the involvement of tissue kallikrein may not be ruled out in the present experiment. The significant increase in the tissue

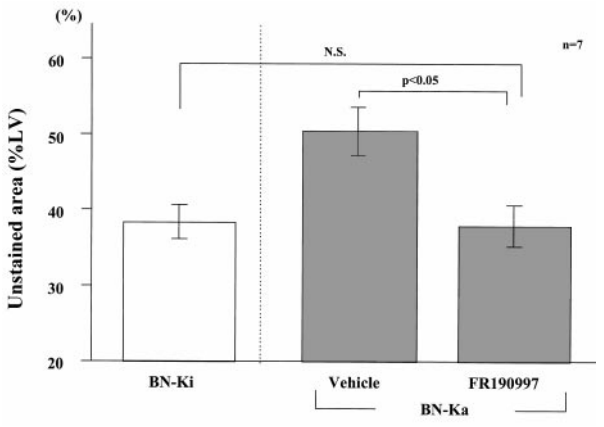

Figure 7 Effect of FR190997, a non-peptide B_2 receptor agonist, on the development of a myocardial infarction in Brown Norway Katholiek rats. Myocardial infarction 24 h after coronary ligation was assessed by triphenyltetrazolium chloride (TTC) staining and expressed as percentage of unstained area of the left ventricle. Values are means with s.e.mean obtained from seven rats. ANOVA was used to evaluate the significance of differences. LV, left ventricle; BN-Ka, Brown Norway Katholiek rats; BN-Ki, Brown Norway Kitasato rats.

levels of BK and BK-(1-5) was not detected in the present experiment. These suggest that generating site of kinins may be restricted to the microcirculation rather than the interstitial tissues of myocardium.

To identify the BK receptors involved in this protective action of endogenous kinins, we administered the B_2 receptor antagonist Hoe140 to rats whose left coronary artery was ligated. The results were essentially the same as those from mutant BN-Ka and those observed in aprotinin-treated rats, suggesting that B_2 receptor signalling was a major pathway for the inhibition of MI in this model. The up-regulation of expression of B_1 receptors was reported in the myocardium after infarction (Tschope *et al*., 2000). However, the lack of effect of des Arg^{10}-[Hoe140], a B_1 antagonist, suggest that the involvement of B_1 receptor signalling may be negligible in the present experiment. It was reported that the cardioprotective action during coronary ischaemia was enhanced by ACE inhibitors, and this enhancement was abolished by bradykinin B_2 receptor antagonists (Ito *et al*.,1997). Our present results also support the significance of B2 receptors without ACE inhibitor treatment.

How endogenous mediators or factors prevented ischaemic injury during coronary occlusion was extensively studied. Several potential factors such as adenosine (Liem *et al*., 2001; Miura *et al*., 2001), heart shock proteins (Hamilton *et al*., 2001), protein kinase C (Sharma & Singh, 2001; Matsumura *et al*., 2000), ATP-sensitive potassium channels (Toombs *et al*., 1993; Menasche *et al*., 1995), nitric oxide (Stefano *et al*., 2001), and prostaglandins (Shinmura *et al*., 2000) were believed to be involved in the preventive action against the development of MI. In addition to the above factors, it is believed that kinin may elicit this preventive action, because of its biological activities in increasing the coronary blood flow when infused directly into the coronary artery. To reveal the protective mechanisms of endogenous kinin, we determined the regional coronary blood flow using microspheres injected into the left ventricle (Kowallik *et al*., 1991). This approach enabled to determine the regional blood flow in small animals such as rats. The blood flow in the non-perfused regions was completely terminated after coronary occlusion in both BN-Ka and BN-Ki, since the blood supply from the left coronary artery was completely blocked. It is, however, quite interesting that, to judge from the significant reduction in the numbers of microspheres detected in BN-Ka and Hoe140-treated rats, the regional blood flow was maintained by endogenous kinin in the non-occluded regions such as those around the necrotic lesion and even in the quadrant opposite the infarction site, whose blood supply was maintained with the intact coronary artery. The endogenous kinins attenuate the MI, possibly by acting on the vasculature in the marginal zone of the area at risk, thus facilitating the blood flow from the non-ligated coronary artery or the marginal zone of the necrotic lesion.

Judging from the present results regarding the regional blood flow, the stimulation of B_2 receptor signalling by coronary application will be expected to reduce the MI and subsequently save the patient from death by cardiac dysfunction. Supplementation of B_2 agonist not only into the occluded coronary artery but also into the patient coronary artery will probably also reduce the size of MI. We previously reported that the first non-peptide B_2 agonist, FR190997, dilated resistance vessels as did BK (Majima *et al*., 2000), but the vasodilating effect of FR190997 *in vivo* was considerably prolonged (Aramori *et al*., 1997; Majima *et al*., 2000). Furthermore, FR1909997 effectively prevented the development of hypertension in young spontaneously hypertensive rats (Majima *et al*., 2000). Therefore, it may be a powerful tool for the investigation of B_2 receptor function and may be useful for therapy during MI. In fact, administration of FR190997 in BN-Ka rats caused significant reduction in the size of MI, in comparison with that in vehicle-treated rats. The size of MI in FR190997-treated BN-Ka rats was the same as that in BN-Ki rats. This suggested that B_2 receptor signalling was the major signalling pathway for the attenuation of MI, and that a stable B_2 receptor agonist, such as FR190997, is a good tool for the treatment of MI.

In conclusion, by using mutant rats and a selective B_2 antagonist, we demonstrated that the endogenous kinins had a capacity for reducing the severity of MI through B_2 receptor signalling. This protective action was attributable to the increase of regional blood flow around the ischaemic lesions. Pharmacological application of agents that can stimulate B_2 receptor signaling may become useful tools for treating MI.

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