British Journal of Pharmacology (2000) 130, 1076–1082

www.nature.com/bjp

Participation of kinins in the captopril-induced inhibition of intimal hyperplasia caused by interruption of carotid blood flow in the mouse

[1,2]Costanza Emanueli, [1,2]Maria Bonaria Salis, [4]Carlos Figueroa, [5]Julie Chao, [5]Lee Chao, [1]Leonardo Gaspa, [2]Maurizio C. Capogrossi & *[,1,3]Paolo Madeddu

[1]Gene Therapy Section of the National Laboratory of the National Institute of Biostructures and Biosystems (I.N.B.B.), Osilo, Sassari, Italy; [2]Laboratorio di Patologia Vascolare, Istituto Dermopatico dell' Immacolata (IDI, IRCCS), Roma, Italy; [3]Institute of Internal Medicine, School of Medicine, University of Sassari, Sassari, Italy; [4]Istituto de Histologia y Patologia, Universidad Austral de Chile, Isla Teja, Valdivia, Chile and [5]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina, U.S.A.

1 In the rat balloon injury model, angiotensin-converting enzyme (ACE) inhibitors prevent vascular remodelling by inhibiting angiotensin II generation and kinin breakdown. We investigated if ACE inhibition also prevents the structural vascular responses to disruption of carotid artery blood flow and if kinin potentiation plays a role in such a protection.

2 Morphometric analysis of the structural alterations caused by ligation of the left carotid artery was performed 14 days after surgery in J129Sv wild-type mice ($B_2^{+/+}$) drinking normal tap water or water containing captopril (120 mg kg^{-1} per day). In addition, the effect of captopril on vascular remodelling was tested in $B_2^{+/+}$ given the bradykinin (BK) B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (DALBK, 50 nmol kg^{-1} per day, intraperitoneally) or the BK B_2 receptor antagonist D-Arg,[Hyp^3,Thi^{5}D-Tic^7,Oic^8]-BK (icatibant, 1 μmol kg^{-1} per day, intraperitoneally), and in B_2 receptor gene knockout mice ($B_2^{-/-}$).

3 Interruption of blood flow resulted in carotid artery intimal hyperplasia and media thickening in untreated $B_2^{+/+}$, these responses being partially suppressed by captopril. The inhibition of intimal thickening exerted by captopril was reduced in $B_2^{+/+}$ given DALBK or icatibant ($P<0.05$ for both comparisons) as well as in $B_2^{-/-}$ ($P<0.05$). Neither antagonism of kinin receptors nor disruption of the B_2 receptor gene altered the suppressive effect of captopril on media thickening. The protection of vascular wall structure was independent of the reduction in blood pressure by captopril.

4 These results demonstrate that kinins participate in the inhibitory effect of captopril on intimal hyperplasia *via* B_1 and B_2 receptor signalling. Our findings may have important implications in treating vascular remodelling evoked by altered shear stress conditions.

British Journal of Pharmacology (2000) **130,** 1076–1082

Keywords: Intimal hyperplasia; angiotensin converting enzyme inhibitors; kinins; receptor antagonists; nitric oxide; knockout mice

Abbreviations: ACE, angiotensin converting enzyme; B_1, BK B_1 receptor; B_2, BK B_2 receptor; $B_2^{-/-}$, B_2 receptor gene knockout mice; $B_2+/+$, wild-type mice; BK, bradykinin; DALBK, des-Arg^9-[Leu^8]-BK; EC, vascular endothelial cell; EEL, external elastic lamina; I, tunica intima; icatibant, D-Arg,[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK; IEL, internal elastic lamina; L-NAME, N-nitro-arginine-L-methyl-ester; M, tunica media; NO, nitric oxide; SBP, systolic blood pressure; SMC, vascular smooth muscle cell

Introduction

Intimal hyperplasia due to neointima formation often causes clinically significant restenosis of arteries that have undergone therapeutic percutaneous transluminar angioplasty (Glagov, 1994). The potential of angiotensin-converting enzyme (ACE) inhibitors in prevention of neointima formation was first addressed in a rat model of carotid artery balloon injury (Powell *et al*., 1989). This protective effect was mainly attributed to inhibition of the ACE-induced generation of angiotensin II a potent vasopressor implicated in vascular smooth muscle cell (SMC) growth (Itoh *et al*., 1993), proliferation (Huckle & Earp, 1994), and migration (Farhy *et al*., 1997). In addition, as ACE is also responsible for kinin breakdown, the possible contribution of kinins in the favourable cardiovascular effects exerted by ACE inhibitors needs to be considered (see Linz *et al*., 1995).

*Author for correspondence; E-mail: madeddu@yahoo.com

The presence of a local kallikrein-kinin system in the vasculature has been firmly established (Madeddu *et al*., 1993; 1994). Kinins are vasoactive peptides released by kallikreins from the precursor kininogens. Binding of kinins to specific 7 trans-membrane domain, G-protein-coupled B_1 and B_2 receptors expressed on vascular endothelial cells (ECs) stimulates the formation of nitric oxide (NO) and prostacyclin, thus leading to activation of cyclic GMP and cyclic AMP pathways (Blaukatt *et al*., 1996; D'Orleans-Juste *et al*., 1989). Differently from the constitutively expressed B_2 receptors, B_1 are inducible receptors, i.e. their expression is activated only under particular pathophysiologic conditions, such as inflammation (see Ahluwalia & Perretti, 1999), hypertension (Emanueli *et al*., 1999), and diabetes (Zuccolo *et al*., 1996). The relevance of B_2 receptors in prevention of vascular remodelling by ACE inhibitors has been demonstrated in the rat carotid balloon injury model by the use of the selective receptor antagonist icatibant (deBlois *et al*., 1992; Farhy *et al*.,

1993). Whether the B_1 receptor subtype is involved in these processes remains unknown.

Recently, a mouse model has been introduced in which vascular wall remodelling is caused by permanent changes in shear stress conditions, as obtained by interruption of carotid artery blood flow (Kumar *et al*., 1997; Kumar & Lindner, 1997; Bryant *et al*., 1999). In this case, vascular endothelium is not removed, thus allowing addressing the importance of substances that are released or targeted to ECs (Langille & O'Donnell, 1986). Furthermore, availability of a murine model of vascular remodelling is essential to exploit the informative potential of gene knockout strategy that is generally carried out in mice.

The aim of the present study was 2 fold: (1) to evaluate if ACE inhibition prevents vascular remodelling induced by disruption of carotid artery blood flow in the mouse and (2) to determine if kinins play a role in this phenomenon *via* activation of B_1 and/or B_2 receptors.

To this purpose, mice underwent ligature of the left carotid artery and were given captopril alone or in combination with B_1 or B_2 receptor antagonists. The preventive effect of captopril on vascular remodelling was also evaluated in mice in which the gene encoding for the B_2 receptor was knocked-out by gene targeting and homologous recombination ($B_2^{-/-}$) (Borkowski *et al*., 1995; Madeddu *et al*., 1997).

Methods

Experiments were conducted using a protocol that was approved by the Institutional Animal Care and Use Committee. All procedures complied with the standards for care and use of animal subjects as stated in the *Guide for the Care and the Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, U.S.A.). Male (2–3 months of age) J129 Sv wild-type mice ($B_2^{+/+}$) were obtained from Jackson Laboratory (Bar Harbor, MN, U.S.A.). $B_2^{-/-}$, generated by gene targeting and homologous recombination on a J129 Sv genetic background (Borkowski *et al*., 1995), were kindly provided by Dr Fred Hess (Merck Laboratories, West Point, PA, U.S.A.).

Surgical procedures

With mice under 2,2,2-tribromoethanol anaesthesia (880 mmol kg^{-1}, intraperitoneally), the left common carotid artery was exposed through a small midline incision of the neck and ligated with 6-0 silk just proximal the bifurcation of the vessel to disrupt blood flow. At the same occasion, osmotic minipumps (Alzet, Alza Co., Palo Alto, CA, U.S.A.) were implanted into the abdomen for continuous intraperitoneal (i.p.) drug delivery. In additional groups of $B_2^{+/+}$ and $B_2^{-/-}$, the carotid artery was exposed, but not ligated (sham-operation).

Experimental protocols

Sham-operated animals were used for reference. Carotid artery-ligated $B_2^{+/+}$ or $B_2^{-/-}$ were given regular tap water to drink or water containing captopril (120 mg kg^{-1} per day for 14 days). In addition, $B_2^{+/+}$ received an i.p. infusion (12 μl per day) of the bradykinin (BK) B_1 receptor antagonist des-Arg^9-[Leu^8]-BK (DALBK, 50 nmol kg^{-1} per day), the BK B_2 receptor antagonist D-Arg, [Hyp^3,Thi^{5}D-Tic^7,Oic^8]-BK (icatibant, 1 μmol kg^{-1} per day), DALBK and icatibant combined (at the doses stated above), or their vehicle (sterile saline). Each group consisted of at least six mice. Selectivity of icatibant for kinin B_2 receptors has been previously reported in the mouse (Madeddu *et al*., 1997; Nsa Allogho *et al*., 1997). DALBK reportedly acts as a selective B_1 receptor antagonist in rat vasculature (Morbidelli *et al*., 1998). Although partial agonistic effect was recognized in the mouse stomach (Nsa Allogho *et al*., 1997), we have found that DALBK, at the dose indicated above, is able to antagonize the hypotensive effect of des-Arg^9-BK in the mouse and is devoid of residual agonistic activity (Paolo Madeddu, unpublished observations).

Additional groups of carotid artery-ligated $B_2^{+/+}$ received the NO synthase inhibitor N-nitro-arginine-L-methyl-ester (L-NAME, 1.4 mmol kg^{-1} per day, orally), or captopril in combination with L-NAME (same dose and route as indicated above). The doses of captopril and L-NAME were chosen according to previous studies (Emanueli *et al*., 1997; Madeddu *et al*., 1997). Each group consisted of at least six mice.

In a separate set of experiments, carotid artery-ligated $B_2^{+/+}$ and $B_2^{-/-}$ ($n=9$ and 7, respectively) were chronically given the Angiotensin II AT_1 receptor antagonist losartan (15 mg kg^{-1} per day, orally).

Haemodynamic measurements

Systolic blood pressure (SBP) was measured in unanaesthetized mice by tail-cuff plethysmography (Visitech System, U.S.A.) before and 14 days after carotid ligation in $B_2^{-/-}$ treated with captopril or vehicle and in $B_2^{+/+}$ treated with vehicle, captopril, or captopril in combination with DALBK, icatibant, or L-NAME. Body weight was recorded at the same occasions.

Morphometric analysis

Fourteen days after carotid artery ligation or sham-operation, mice were anaesthetized and perfusion fixed at 100 mmHg with 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.3). The whole left common carotid artery was excised and placed for 24 h in 4% paraformaldehyde. Vessels were then processed for paraffin embedding. Five serial sections

Table 1 Tail-cuff systolic blood pressure of $B_2^{+/+}$ or $B_2^{-/-}$ under basal conditions (Basal SBP) and after 14 days from cartoid ligation (Final SBP)

Strain	*Treatment*	*Basal SBP* (mmHg)	*Final SBP* (mmHg)
$B_2^{+/+}$	vehicle	112±4	109±3
$B_2^{+/+}$	captopril	107±2	70±4*
$B_2^{+/+}$	captopril+DALBK	108±2	72±4*
$B_2^{+/+}$	captopril+icatibant	109±3	71±2*
$B_2^{+/+}$	captopril+L-NAME	109±2	85±4*
$B_2^{-/-}$	vehicle	130±4#	127±3#
$B_2^{-/-}$	captopril	128±5	72±5*

Values are means±s.e.mean. Changes in blood pressure reflect the effects of carotid ligation and treatment with various drugs. Carotid artery-ligated mice were given tap water to drink (vehicle) or water containing captopril (120 mg kg^{-1} per day). In addition, wild-type controls receifved the bradykinin (BK) B_1 receptor antagonist des-Arg^9-Leu^8-BK (DALBK, 50 nmol kg^{-1} per day, by i.p. infusion) or the BK B_2 receptor antagonist D-Arg,[Hyp^3, Thi^5 D-Tic^7,Oic^8]-BK (inatibant, 1 μmol kg^{-1} per day, by i.p. infusion), or the NO syntase inhibitor N-nitro-arginine-L-methyl-aster (L-NAME, 1.4 mmol kg^{-1} per day, orally). $B_2^{-/-}$, B_2 receptor gene knockout mice; $B_2^{+/+}$, wild-type controls. #$P<0.05$ *vs* $B_2^{+/+}$ at the same time point within each group treatment, *$P<0.05$ *vs* basal. Each group consisted of at least six mice.

(200 μm apart) of 3 μm thickness were cut starting from 1 mm below the carotid artery bifurcation and proceeding to the aortic arch. Sections were stained with haematoxylin-eosin. Morphometric analysis was performed using a dedicated software package (KS300, Zeiss). The area of tunica intima (I) was recognized as the area delimited by the luminal circumference and the internal elastic lamina (IEL). The area of tunica media (M) was recognized as the area delimited by IEL and the external elastic lamina (EEL). In addition to I and M areas, the length of IEL and EEL were measured. IEL was taken as an index of inward remodelling, and EEL as a measure of vascular constriction. The total cell number per I and M cross section was evaluated at a magnification of 1000 × using a calibrated grid. Then, I and M cellular density was calculated by dividing total cell count by the respective area. For each carotid artery, the values obtained from the five sections were averaged.

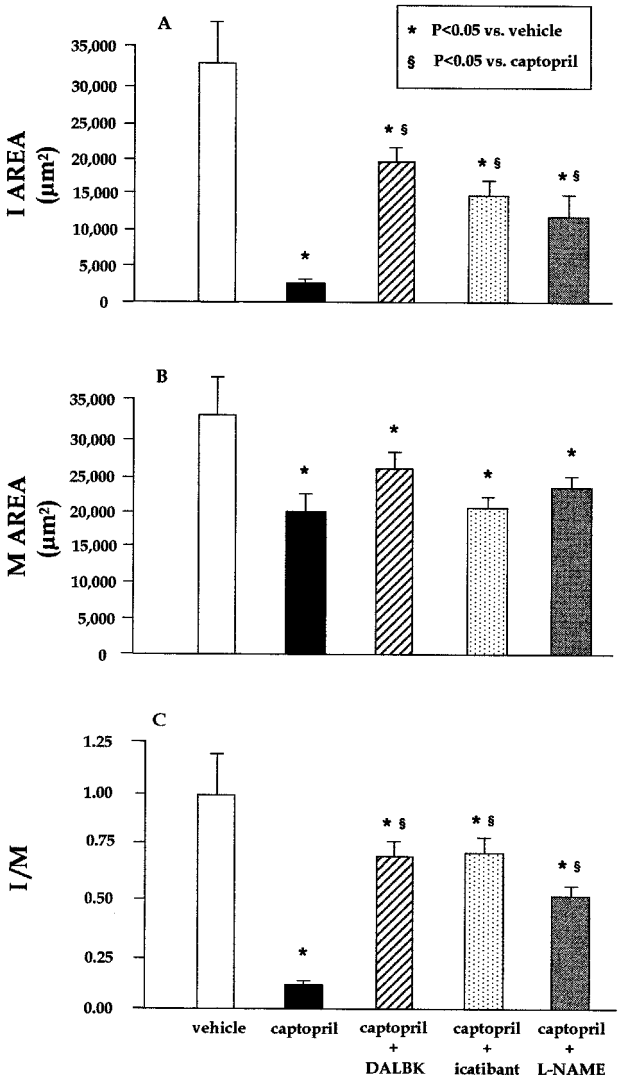

Figure 1 Bar graphs show the areas of tunica intima (I, A) and media (M, B) of the left common carotid artery and the ratio between I and M areas (C) at 14 days from disruption of blood flow in wild-type J129 Sv mice ($B_2^{+/+}$) given vehicle (normal drinking water; $n=12$), captopril (120 mg kg^{-1} per day in the drinking water; $n=7$), captopril plus the B_1 receptor antagonist des-Arg^9-[Leu^8]-bradykinin (DALBK) (50 nmol kg^{-1} per day, i.p.; $n=7$), captopril plus the B_2 receptor antagonist D-Arg,[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (icatibant, 1 μmol kg^{-1} per day, i.p.; $n=7$), or captopril plus the nitric oxide synthase inhibitor, N-nitro-L-arginine-methyl-ester (L-NAME, 1.4 mmol kg^{-1} per day in the drinking water, $n=7$). Values are means ± s.e.mean.

Drugs

Captopril, L-NAME, and DALBK were purchased from Sigma (Milan, Italy) and losartan from Merck (Milan, Italy). Icatibant was a generous gift of Hoechst Pharmaceutical Co. (Frankfurt, Germany).

Statistical analysis

Data are expressed as mean ± s.e.mean. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons among independent groups in which ANOVA and P test indicated significant differences, the statistical value was determined according to Bonferroni's method. Differences within and between groups were determined using paired or unpaired Student's t-test, respectively. A probability (P) value less 0.05 was considered significant.

Results

Haemodynamic measurements

Captopril did not affect the surviving rate or the gain in body weight of $B_2^{+/+}$ or $B_2^{-/-}$ (data not shown).

As shown in Table 1, basal SBP was higher in $B_2^{-/-}$ compared to $B_2^{+/+}$ ($P<0.05$). This difference between strains persisted after carotid artery ligation in vehicle-treated mice. Captopril significantly decreased SBP levels in carotid artery-ligated $B_2^{-/-}$ and $B_2^{+/+}$, so that after 14 days of ACE inhibitor treatment no significant difference was observed between the two strains. In $B_2^{+/+}$, the hypotensive effect of captopril was not altered by the administration of kinin receptor antagonists or L-NAME.

Morphometric analysis

In vehicle-treated $B_2^{+/+}$, disruption of carotid artery blood flow determined a reduction in vessel lumen area by a combination of I hyperplasia (I area: 32,685 ± 7648 μm^2 *vs* not measurable at the same magnification in sham-operated mice), M thickening (M area: 32,891 ± 4361 *vs* 21,520 ± 5368 μm^2 in sham-operated mice, $P<0.05$), and reduction in IEL and EEL lengths (975 ± 143 *vs* 1281 ± 141 μm and 1102 ± 75 *vs* 1380 ± 171 μm in sham-operated mice, respectively, $P<0.05$ for both comparisons).

As shown in Figure 1, in $B_2^{+/+}$ captopril inhibited I ($P<0.001$) and M ($P<0.05$) hyperplasia, leading to reduction in I/M ratio (0.13 ± 0.01 *vs* 0.97 ± 0.22 in vehicle-treated mice, $P<0.01$). Captopril did not alter the effect of carotid artery ligature on IEL or EEL length (data not shown).

In vehicle-treated $B_2^{+/+}$, chronic administration of DALBK or icatibant alone did not change the vascular remodelling response to carotid artery ligation (data not shown). However, as shown in Figure 1A, either B_1 or B_2 receptor antagonism partially prevented the inhibition of I thickening exerted by captopril ($P<0.05$ for both comparisons). The combined administration of DALBK and icatibant did not further reduce the captopril-induced inhibition of I hyperplasia (I area: 25,985 ± 6040, P = N.S. *vs* captopril plus DALBK or captopril plus icatibant).

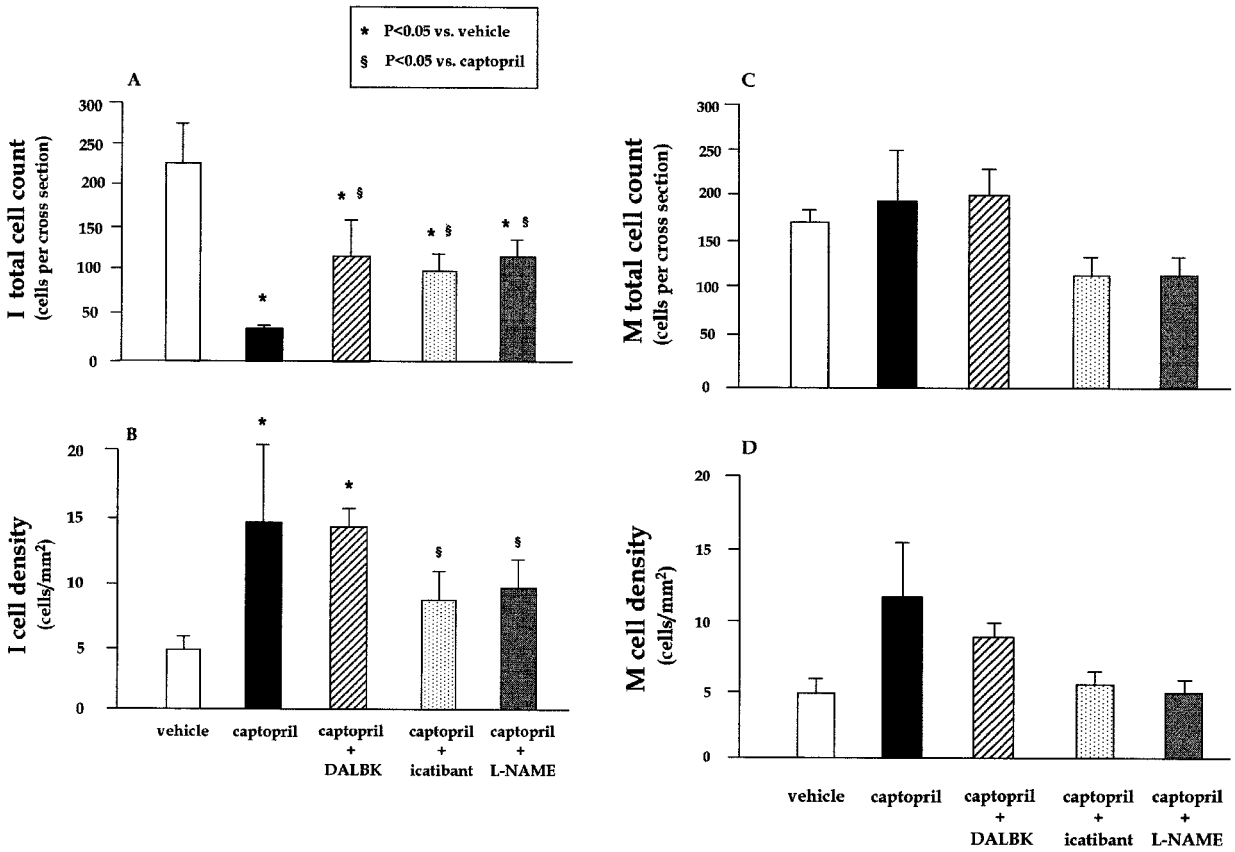

Figure 2 Bar graphs show the total cell count per cross section and the cell density in tunica intima (I, A,B) and media (C,D) at 14 days from disruption of carotid artery blood flow in $B_2^{+/+}$ given vehicle ($n=12$), captopril ($n=7$), captopril plus DALBK ($n=7$), captopril plus icatibant ($n=7$), or captopril plus L-NAME ($n=7$). Values are means$\pm$s.e.mean.

In contrast, the captopril-induced-suppression of M thickening was not altered by DALBK, icatibant (Figure 1B), or the two antagonists in combination (data not shown).

L-NAME alone did not affect the vascular response to carotid artery ligation in vehicle-treated $B_2^{+/+}$ (data not shown), but it reduced the inhibition of I thickening exerted by captopril (Figure 1A). L-NAME did not change captopril-induced effect on M hyperplasia (Figure 1B).

In carotid artery-ligated $B_2^{+/+}$, captopril reduced total cell count per I cross section (35 ± 3 *vs* 224 ± 65 cells in vehicle-treated mice, $P<0.001$, Figure 2A), and increased I cell density (15 ± 6 *vs* 5 ± 1 cells/mm^2 in vehicle-treated mice, $P<0.05$, Figure 2B). In contrast, as shown in Figure 2C, D, captopril produced borderline changes in total cell count per M cross section ($P=0.07$) or cell density ($P=0.09$). Either DALBK or icatibant or L-NAME antagonized captopril-induced effect on I cell number ($P<0.05$ for all comparisons, Figure 2A), but only icatibant and L-NAME antagonized the effect on I cell density ($P<0.05$, Figure 2B).

Figure 3 shows the typical remodelling response to interruption of blood flow and the preventive action of captopril against I and M hyperplasia. In addition, the effects of B_1 or B_2 antagonism on captopril-induced protection against I thickening are shown in the same figure.

Next, we examined the characteristics of carotid artery structure in mice with genetic disruption of the B_2 receptor. No morphologic difference was seen when comparing sham-operated carotid arteries of $B_2^{-/-}$ with those of $B_2^{+/+}$ (data not shown). In addition, as shown in Figure 4, the absence of the B_2 receptor gene did not affect the vascular wall response to carotid artery ligation in vehicle-treated animals (Figure 4). However, as shown in Figure 4A, the null mutation was associated with a significant reduction in captopril-induced preventive effect on I hyperplasia (I area: 8381 ± 593 *vs* 2459 ± 250 μm^2 in $B_2^{+/+}$, $P<0.05$). By contrast, inhibition of M thickening by captopril was similar in $B_2^{-/-}$ and $B_2^{+/+}$ (Figure 4B). As a consequence, I/M ratio was higher in $B_2^{-/-}$ compared to $B_2^{+/+}$ ($P<0.05$).

In carotid artery-ligated $B_2^{-/-}$, captopril reduced total cell count per I cross section (95 ± 3 *vs* 179 ± 16 cells in vehicle-treated mice, $P<0.01$) and increased I cell density (12 ± 1 *vs* 7 ± 1 cells/mm^2 in vehicle-treated mice, $P<0.05$).

Finally, we evaluated the effect of AT_1 receptor antagonism on vascular remodelling. Losartan inhibited I and M hyperplasia similarly in $B_2^{+/+}$ and $B_2^{-/-}$ (data not shown). In addition, the antagonist decreased I cell number per cross section and increased I cell density with no difference between strains (data not shown). Cell number per M cross section did not change, while M cell density decreased following losartan treatment (data not shown) with no differences between strains.

Discussion

At the best of our knowledge, this is the first study demonstrating a preventive action of ACE inhibition in a mouse model of I hyperplasia. The importance of B_2 receptor signalling in this phenomenon has been revealed by pharmacological blockade or genetic disruption of the receptor. Another novel discovery is that also B_1 receptors participate in the suppressive effect of ACE inhibition on I hyperplasia.

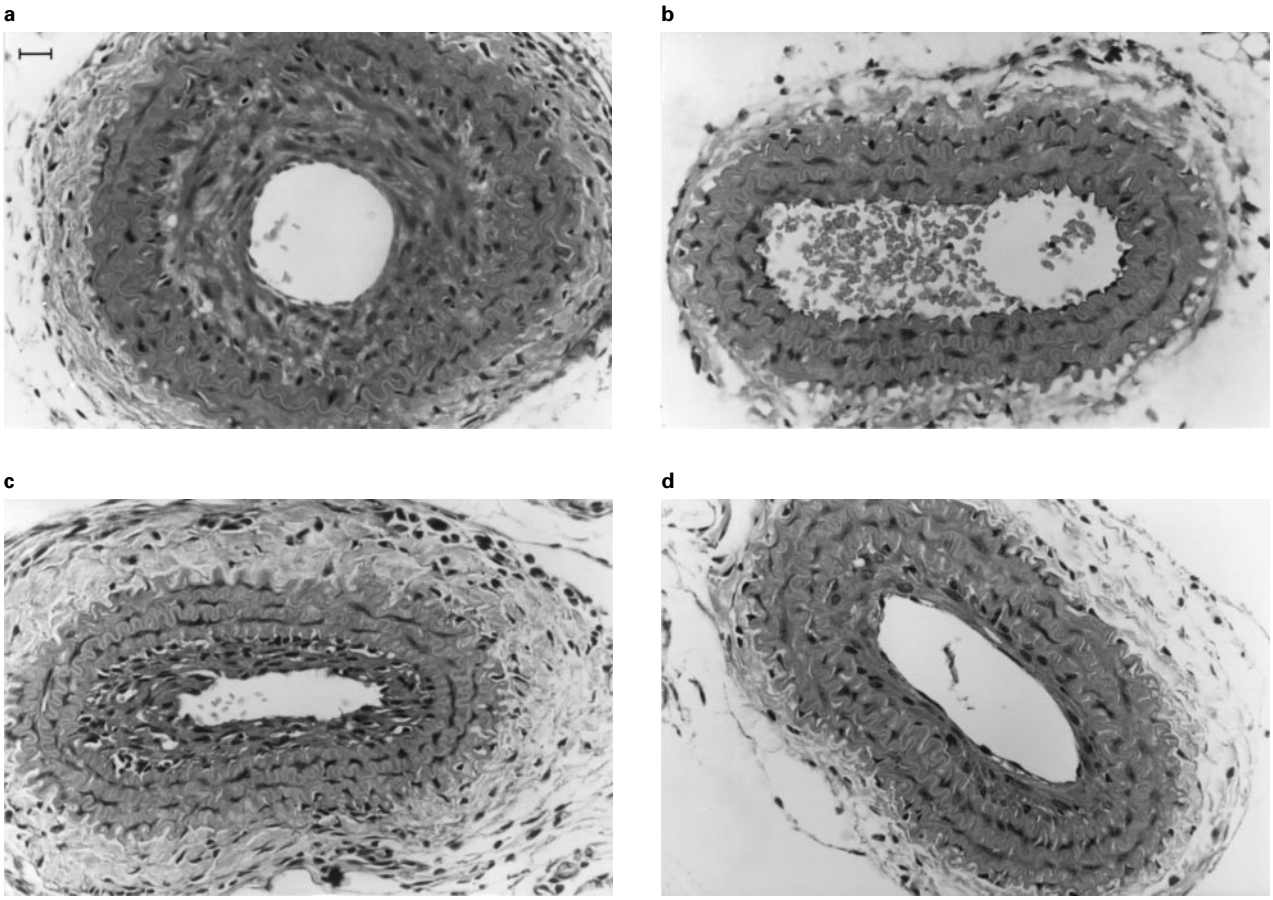

Figure 3 Pictures show representative haematoxilin and eosin-stained left common carotid artery transverse sections of $B_2^{+/+}$ treated with vehicle (A), captopril (B), captopril in combination with the B_1 receptor antagonist DALBK (C), or captopril in combination with the B_2 receptor antagonist icatibant (D). Vessels were taken 14 days after blood flow interruption. Sections were obtained at 1 mm from carotid artery bifurcation. Scale bar corresponds to 10 μm.

Implication of kinins in the preventive effect of ACE inhibitors against neointima formation has been previously reported in the rat (deBlois *et al*., 1992; Farhy *et al*., 1993). In the above studies, arterial remodelling was triggered by endothelium removal using a balloon catheter. However, development of intimal lesions in atherosclerosis models and in human vascular disease occurs in the absence of noticeable endothelial denudation at sites of altered haemodynamics associated with low shear stress. Therefore, we thought it would be worthwhile to address the role of kinin receptors in a murine model of vascular remodelling attributable to altered shear stress conditions (Kumar & Lindner, 1997). Attention was focused on the B_1 receptor in consideration of the mounting importance of this subtype receptor in inflammatory-like conditions (see Ahluwalia & Perretti, 1999). Induction of B_1 receptor-dependent vasoconstriction following balloon catheter injury reportedly occurs in the rabbit carotid artery (Pruneau *et al*., 1994). However, the overall relevance of these changes to vascular remodelling remains unclear. We have found that B_1 receptor antagonism does not alter the remodelling response to interruption of carotid artery blood flow, but prevents in part the inhibitory effect of captopril against I hyperplasia. These results indicate a role for the B_1 receptor signalling in the preventive effect of ACE inhibitors against I thickening. The functional importance of B_1 receptors might have been enhanced by ACE inhibition because of a shift of kinin degradation through kininase I in the direction of des-Arg^9-derivatives (Israel & Saavedra, 1987; Lamontagne *et al*., 1995). Immunohistochemistry studies have clearly demonstrated the presence of inflammatory cells (macrophages and monocytes) in the adventitia, developing intima, and near the luminar surface of carotids that undergo blood flow disruption (Kumar & Lindner, 1997). In this inflammatory milieu, the B_1 receptor may be up-regulated, thus enabling cells expressing this receptor subtype to act as magnets for kinin peptides augmented by ACE inhibition.

While knockout of the B_1 receptor is still awaited, availability of null mutant $B_2^{-/-}$ has allowed to determine the impact of the absence of B_2 receptors on the development of cardiovascular phenotype (Madeddu *et al*., 1997). We found that this genetic defect does not result in abnormalities of the structure of carotid artery under basal conditions or in altered vascular remodelling responses to carotid artery ligature. However, according to our results a functional B_2 receptor signalling participates in the inhibition of I hyperplasia induced by captopril. Recently, the hypothesis that ACE inhibitors exert beneficial cardiovascular effects by blocking B_2 receptor desensitization has been advanced (Minshall *et al*., 1997). Whether such an intriguing mechanism is relevant in the protection against I thickening remains to be determined.

The near-stasis conditions in more distal segment of the ligated carotid artery may result in activation of extrinsic coagulation pathway. Therefore, we cannot exclude that kinins released by the plasma kallikrein from high molecular weight kininogen might contribute together with vascular tissue kinins to the protective effect of captopril. However, as previously shown by

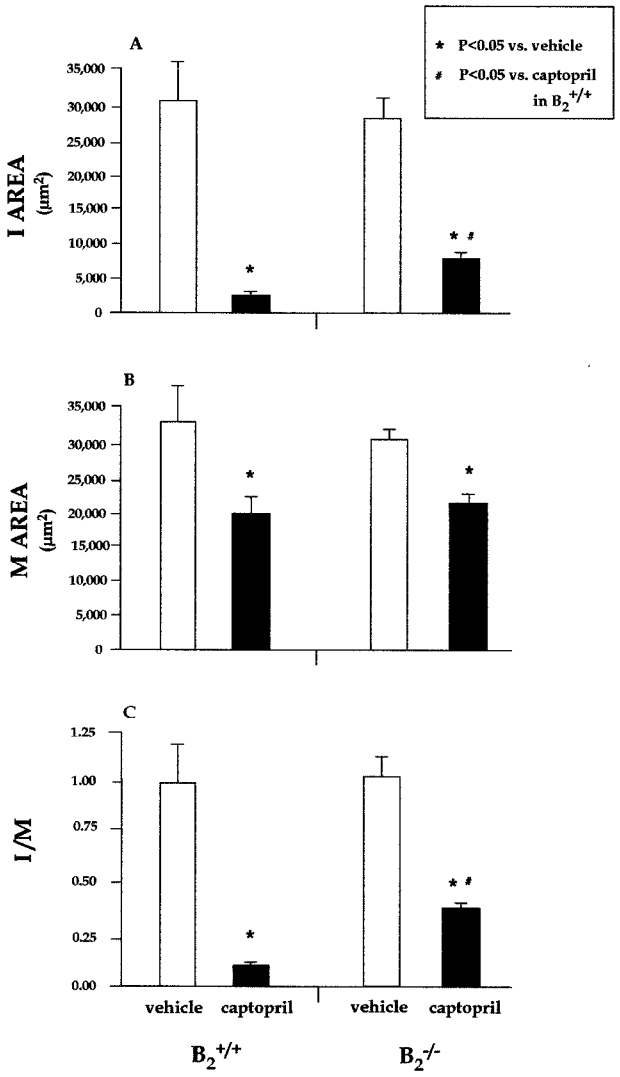

Figure 4 Bar graphs show the areas of left common carotid artery tunica intima (I, A) and media (M, B), and the ratio between I and M areas (C) at 14 days from disruption of blood flow in wild-type ($B_2^{+/+}$, left) and B_2 receptor gene knockout mice ($B_2^{-/-}$, right) given vehicle ($B_2^{+/+}$: $n=12$, $B_2^{-/-}$: $n=10$) or captopril ($B_2^{+/+}$: $n=7$, $B_2^{-/-}$: $n=10$). Values are means ± s.e.mean.

Kumar & Lindner (1997), preservation of endothelium maintains a non-thrombotic surface in this model.

Consistent with previous results (Emanueli *et al*., 1997), we found that neither disruption nor pharmacological blockade of the B_2 receptor altered the blood pressure fall in response to ACE inhibition in mice. Therefore, it appears unlikely that systemic haemodynamic changes are responsible for the different structural vascular responses observed among groups given captopril. Furthermore, in our model the driving factor for vascular remodelling is constituted by the permanent alterations in shear stress resulting from cessation of blood flow, and not by systemic blood pressure.

Participation of kinins in captopril-induced protective effects appears to be restricted to inhibition of I hyperplasia, since the suppressive action of ACE inhibition on medial thickening was not antagonized by manipulation of kinin receptors. Moreover, kinin blockade inhibits only in part captopril-induced protection against I hyperplasia. These findings are in line with the fundamental role of Angiotensin II in vascular remodelling as indicated by our results in losartan-treated animals.

ACE inhibition decreased I total cell count with the participation of both B_1 and B_2 receptors in this phenomenon. By contrast, M total cell count was not affected by captopril either alone or in combination with receptor antagonists. Taken together these results suggest that increased kinin levels by captopril could inhibit SMC proliferation. This is in accordance with recent findings from our group showing that kinins released by tissue kallikrein inhibit SMC proliferation (Murakami *et al*., 1999). *In vitro* experiments indicate that binding of kinins to aortic SMC receptors stimulates prostacyclin formation, thus leading to increased cyclic AMP levels and subsequent inhibition of SMC proliferation (Dixon *et al*., 1990). The mechanism by which increased kinin levels inhibit vascular growth *in vivo* may also involve the induction and/or activation of NO synthase, and in fact L-NAME diminished the inhibition of I hyperplasia exerted by captopril in our experimental setting as well as in the rat balloon injury model (Farhy *et al*., 1993). Binding of kinins to vascular endothelial B_2 receptors increases NO release (Zhang *et al*., 1997). Once released by ECs, NO reaches SMC where it activates guanilate cyclase, thus increasing the production of cyclic GMP, a potent inhibitor of SMC proliferation and migration (Cornwell *et al*., 1994; Grag & Hassid, 1989; Sarkar *et al*., 1996). Accordingly, Farhy *et al*. (1997) showed that kinin-stimulated NO release contributes to the beneficial effect of ACE inhibitors in the rat balloon injury model by reducing SMC migration from the tunica media to the I. As already stated above, we found that M cell count was unchanged by captopril. We would expect that if ACE inhibition blocked SMC migration to I without affecting proliferation rate, the total number of cell would be increased in the tunica media. Since this was not the case, it is likely that both SMC migration and proliferation were inhibited by captopril. Kinins have been demonstrated to attenuate programmed cell death in ischaemic tissue (Yoshida *et al*., 2000). Since hypoxia-induced apoptosis occurrs in the early phases of vascular remodelling after carotid artery ligation (Kumar & Lidner, 1997), it is possible that an anti-apoptotic effect of kinins may in part balance the kinin-induced inhibitory effect on SMC proliferation. Further studies using markers of cellular turnover (i.e. cell proliferation and apoptosis) may help address this issue.

The finding that I cell density was augmented in captopril-treated group, with this effect being antagonized by icatibant, favours a suppressive role of kinins, *via* the B_2 receptor, against matrix production and/or deposition. This is in accordance with *in vitro* experiments demonstrating that BK down-regulates extracellular matrix protein production *via* NO and cyclic GMP (Kim *et al*., 1999) and with a report showing that kinin B_2 receptor antagonism enhances the spontaneous interstitial deposition of collagen in response to myocardial infarction in the rat (Wollert *et al*., 1997). An alternative explanation for kinin-mediated effects of captopril on cell density would be a decrease in cell size.

In conclusion, we have demonstrated that endogenous kinins acting on both their receptor subtypes play an important role in the preventive effect of ACE inhibition against I hyperplasia in a mouse carotid artery model in which vascular remodelling is induced by cessation of blood flow. These findings underline the importance of the kallikrein-kinin system in vascular biology and may have important implications in treating I hyperplasia evoked by altered shear stress conditions.

The financial support of Telethon-Olnus (grant A.105) is gratefully acknowledged. This study was supported in part by National Institutes of Health (Grants HL29397 and HL52196) Biomed 96-

1160 and Regione Autonoma Della Sardegna (assessorato Della Pubblica Istruzione). In addition, we would like to thank Dr Elena Cigola from the University of Parma for the expert advices in histologic procedures and Dr Renzo Filippetti, Mr Vittorio Lelii, and Mr Leandro Travaglini from the Universtià Cattolica del Sacro Cuore (Rome, Italy) for their assistance in the animal care.

References

AHLUWALIA, A. & PERRETTI, M. (1999). B_1 receptors as a new inflammatory target. Could this B the 1? *TiPS.,* **20,** 100–104.

BLAUKATT, A., ABD ALLA, S., LOHSE, M.J. & MULLER-ESTERL, W. (1996). Ligand-induced phosphorilation:dephosphorylation of the endogenous bradykinin B_2 receptor from human fibroblast. *J. Biol. Chem.,* **271,** 32366–32374.

BORKOWSKI, J.A., RANSON, R.W., SEABROOK, G.R., TRUMBAUER, M., CHEN, H., HILL, R.G., STRADER, C.D. & HESS, J.F. (1995). Targeted disruption of bradykinin B_2 receptor gene in mice eliminates bradykinin action in smooth muscle and neurons. *J. Biol. Chem.,* **270,** 13706–13710.

BRYANT, S.R., BJERCKE, R.J., ERICHESEN, D.A., REGE, A. & LINDNER, V. (1999). Vascular remodeling in response to altered blood flow is mediated by fibroblast growth factor-2. *Circ. Res.,* **84,** 323–328.

CORNWELL, T.L., ARNOLD, E., BOERTH, N.J. & LINCOLN, T.M. (1994). Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am. J. Physiol.,* **276,** C1405–C1413.

DEBLOIS, D., LOMBARDI, D.M., GARVIN, M.A. & SCHWARTZ, S.M. (1992). Inhibition by ramipril of intimal hyperplasia in the denuded rat carotid is reversed by Hoe 140, a kinin B_2 receptor antagonist. *Circulation,* **86,** I226(Abstr.).

DIXON, B.S., BRECKON, R., FORTUNE, J., VAVREK, R.J., STEWART, J.M., MARZEC-CALVERT, R. & LINAS, S.L. (1990). Effects of kinins on cultured arterial smooth muscle. *Am. J. Physiol.,* **258,** C299–C308.

D'ORLEANS-JUSTE, P., DENUCCI, G. & VANE, J.R. (1989). Kinins act on B_1 or B_2 receptors to release conjointly endothelium-derived relaxing factor and prostacyclin from bovine aortic endothelial cells. *Br. J. Pharmacol.,* **96,** 920–926.

EMANUELI, C., CHAO, J., REGOLI, D., CHAO, L., NI, A. & MADEDDU, P. (1999). The bradykinin B_1 receptor and the central regulation of blood pressure in spontaneously hypertensive rats. *Br. J. Pharmacol.,* **126,** 1769–1776.

EMANUELI, C., RONDONI ANGIONI, G., ANANIA, V., SPISSU, A. & MADEDDU, P. (1997). Blood pressure responses to acute or chronic captopril in mice with disruption of bradykinin B_2-receptor gene. *J. Hypertens.,* **15,** 1701–1706.

FARHY, D.R., CARRETERO, O.A., HO, K.L. & SCICLI, A.G. (1993). Role of kinins and nitric oxide in the effects of ACE inhibition on neointima formation. *Circ. Res.,* **72,** 1202–1210.

FARHY, D.R., PETERSON, E. & SCICLI, A.G. (1997). Kinins and the events influenced by an angiotensin-converting enzyme inhibitor during neointima formation in the rat carotid artery. *J. Hypertens.,* **15,** 421–429.

GLAGOV, S. (1994). Intimal hyperplasia, vascular remodeling, and the restenosis problem. *Circulation,* **89,** 2888–2891.

GRAG, U.C. & HASSID, A. (1989). Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J. Clin. Invest.,* **83,** 1774–1777.

HUCKLE, W.R. & EARP, H.S. (1994). Regulation of cell proliferation and growth by angiotensin II. *Prog. Growth Factor Res.,* **5,** 177–194.

ISRAEL, A. & SAAVEDRA, J.M. (1987). High angiotensin converting enzyme (kininase II) activity in the cerebrospinal fluid of spontaneously hypertensive rats. *J. Hypertens.,* **5,** 355–357.

ITOH, H., MUKOYAMA, M., PRATT, R.E., GIBBONS, G.H. & DZAU, V.J. (1993). Multiple autocrine growth factors modulate vascular smooth muscle growth response to angiotensin II. *J. Clin. Invest.,* **91,** 2268–2274.

KIM, N.N., VILLEGAS, S., SUMMEROUR, S.R. & VILLAREAL, F.J. (1999). Regulation of cardiac fibroblast extracellular matrix production by bradykinin and nitric oxide. *J. Mol. Cell. Cardiol.,* **31,** 457–466.

KUMAR, A., HOOVER, J.L., SIMMONS, C.A., LINDNER, V. & SHEBUSKI, R.J. (1997). Remodeling with neointima formation in the carotid artery of normal and P-selectin-deficient mice. *Circulation,* **96,** 4333–4342.

KUMAR, A. & LINDNER, V. (1997). Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 2238–2244.

LAMONTAGNE, D., NADEAU, R. & ADAM, A. (1995). Effect of enalaprilat on bradykinin and des-Arg^9-bradykinin release following reperfusion of the ischemic heart. *Br. J. Pharmacol.,* **115,** 476–478.

LANGILLE, B.L. & O'DONNELL, F. (1986). Reduction in arterial diameter produced by chronic decreases in flow are endothelium-dependent. *Science,* **231,** 405–407.

LINZ, W., WIEMER, G., GOHLKE, P., UNGER, T. & SCHOLKENS, B.A. (1995). Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol. Rev.,* **47,** 25–49.

MADEDDU, P., GHERLI, T., BACCIU, P.P., MAIOLI, M. & GLORIOSO, N. (1993). A kallikrein-like enzyme in human vascular tissue. *Am. J. Hypertens.,* **6,** 344–348.

MADEDDU, P., VARONI, M.V., DEMONTIS, M.P., FATTACCIO, M.C., PARPAGLIA, P.P. & GLORIOSO, N. (1994). A kallikrein-like enzyme in the aorta of normotensive and hypertensive rats. *Hypertension,* **23,** 899–902.

MADEDDU, P., VARONI, M.V., PALOMBA, D., EMANUELI, C., DEMONTIS, M.P., GLORIOSO, N., DESSI' FULGHERI, P., SARZANI, R. & ANANIA, V. (1997). Cardiovascular phenotype of a mouse strain with disruption of bradykinin B_2 receptor gene. *Circulation.,* **96,** 3570–3578.

MINSHALL, R.D., ERDOS, E.G. & VOGEL, S.M. (1997). Angiotensin-converting enzyme inhibitors potentiate bradykinin inotropic effects independently of blocking its inactivation. *Am. J. Cardiol.,* **80,** 132A–136A.

MORBIDELLI, L., PARENTI, A., GIOVANNELLI, L., GRANGER, H.J., LEDDA, F. & ZICHE, M. (1998). B_1 receptor involvement in the effect of bradykinin on venular endothelial cell proliferation and potentiation of FGF-2 effects. *Br. J. Pharmacol.,* **124,** 1286–1292.

MURAKAMI, H., YAYAMA, K., MIAO, R.Q., WANG, C., CHAO, L. & CHAO, J. (1999). Kallikrein gene delivery inhibits vascular smooth muscle cell growth and neointima formation in rat artery after balloon angioplasty. *Hypertension,* **34,** 164–170.

NSA ALLOGHO, S., GOBEIL, F., PHENG, L.H., NGUYEN-LE, X.K., NEUGEBAUER, W. & REGOLI, D. (1997). Antagonists for kinin B_1 and B_2 receptors in the mouse. *Can. J. Physiol. Pharmacol.,* **75,** 558–562.

POWELL, J.S., CLOZEL, J.P., MULLER, R.K.M., KUHN, H., HEFTI, F., HOSANG, M. & BAUMGARTNER, H.R. (1989). Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science,* **245,** 186–188.

PRUNEAU, D., LUCCARINI, J.M., ROBERT, C. & BELICHARD, P. (1994). Induction of kinin B_1 receptor-dependent vasoconstriction following balloon catheter injury to the rabbit carotid artery. *Br. J. Pharmacol.,* **111,** 1029–1034.

SARKAR, R., MEINBERG, E.G., STANLEY, J.C., GORDON, D. & WEBB, R.C. (1996). Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. *Circ. Res.,* **78,** 225–230.

WOLLERT, K.C., STUDER, R., DOERFER, K., SCHIEFFER, E., HOLUBARSCH, C., JUST, H. & DREXLER, H. (1997). Differential effects of kinins on cardiomyocyte hypertrophy and interstitial collagen matrix in the surviving myocardium after myocardial infarction in the rat. *Circulation,* **95,** 1910–1917.

YOSHIDA, H., ZHANG, J.J., CHAO, L. & CHAO, J. (2000). Kallikrein gene delivery attenuates myocardial infarction and apoptosis after myocardial ischemia and reperfusion. *Hypertension,* **35,** 25–31.

ZHANG, X., SCICLI, G.A., XU, A., NASJLETTI, A. & HINTZE, T.H. (1997). Role of endothelial kinins in control of coronary nitric oxide production. *Hypertension,* **30,** 1105–1111.

ZUCCOLO, A., NAVARRO, M. & CATANZARO, O. (1996). Effects of B_1 and B_2 receptor antagonists in diabetic mice. *Can. J. Physiol. Pharmacol.,* **74,** 586–589.

(Received November 8, 1999
Revised February 14, 2000
Accepted March 24, 2000)