

Adenovirus-Mediated Human Tissue Kallikrein Gene Delivery Inhibits Neointima Formation Induced by Interruption of Blood Flow in Mice

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Abstract—Tissue kallikrein cleaves kininogen to produce vasoactive kinin peptides. Binding of kinins to bradykinin B₂ receptors on vascular endothelial cells stimulates the release of nitric oxide and prostacyclin, thus activating the cGMP and cAMP pathways. In this study, we evaluated the effects of adenovirus-mediated human tissue kallikrein gene (Ad.CMV-CHK) delivery in a mouse model of arterial remodeling induced by permanent alteration in shear stress conditions. Mice underwent ligation of the left common carotid artery and were injected intravenously with saline or 1.8×10⁹ plaque-forming units of Ad.CMV-CHK or control virus (Ad.CMV-LacZ). Fourteen days after surgery, morphometric analysis revealed that Ad.CMV-CHK reduced neointima formation by 52% ($P<0.05$) compared with Ad.CMV-LacZ. Expression of human tissue kallikrein (HK) mRNA was detected in mouse carotid artery, aorta, kidney, heart, and liver, and recombinant HK was present in the urine and plasma of mice receiving HK gene. Kallikrein gene transfer resulted in increases in urinary kinin, cGMP, and cAMP levels. The protective action of Ad.CMV-CHK on neointima formation was significantly reduced ($P<0.05$) in mice with knockout of the kinin B₂ receptor gene compared with wild-type control mice (J129Sv mice). In contrast, the effect of Ad.CMV-CHK was amplified ($P<0.05$) in transgenic mice overexpressing human B₂ receptor compared with wild-type control mice (c57/Bl6 mice). Thus, the inhibitory effect of recombinant kallikrein on structural alterations caused by the interruption of blood flow appears to be mediated by the B₂ receptor. These results provide new insight into the role of the tissue kallikrein-kinin system in vascular remodeling and suggest the application of HK gene therapy to treat restenosis and atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2000;20:1459-1466.)

Key Words: human tissue kallikrein ■ gene delivery ■ neointima formation ■ bradykinin B₂ receptors ■ mice, transgenic and knockout

Restenosis is one of the major complications of percutaneous transluminal angioplasty and can be regarded as a combination of neointima (NI) formation and arterial remodeling triggered by vascular injury. Vascular remodeling also occurs as an adaptive phenomenon in response to chronic hemodynamic alterations aimed at maintaining a predetermined level of shear stress by permanent modifications in vascular geometry.¹⁻³ The endothelium is considered a critical mediator of the flow-dependent remodeling process.² In fact, vascular endothelial cells (VECs), acting as sensors of intraluminal mechanical forces, release growth factors and vasoactive substances able to induce cell proliferation, migration, and death as well as matrix deposition.²

The presence of a local kallikrein-kinin system in the vasculature is firmly established,⁴⁻⁶ and evidence is now emerging regarding the possible participation of this system

in vascular remodeling. Tissue kallikrein is a serine protease that cleaves low molecular weight kininogen to produce kinin peptides. Kinins stimulate the release of nitric oxide (NO) and prostacyclin (PGI₂) through the activation of bradykinin (BK) B₂ receptors expressed by VECs. NO and PGI₂ exert antiproliferative and antimigratory effects on vascular smooth muscle cells (VSMCs) by increasing intracellular cGMP and cAMP, respectively.⁷⁻¹¹

The contribution of kinins in the protective effect of angiotensin-converting enzyme (ACE) inhibitors against arterial thickening caused by balloon injury was demonstrated in rats by the use of a B₂ receptor antagonist.¹²⁻¹⁵ Recently, adenovirus-mediated human tissue kallikrein (HK) gene delivery proved to be an efficient strategy to increase local or circulating kinin levels for a limited period of time. By this mechanism, HK gene therapy reportedly prevents the devel-

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opment of hypertension, cardiac hypertrophy, and renal failure^{16–18} and suppresses NI formation in balloon-injured rat arteries.¹⁹ Whether the beneficial effect of HK gene therapy can be extended to other models of vascular remodeling remains unknown. Recently, Kumar, Lindner, and colleagues^{20,21} have shown that NI formation can be induced in the mouse carotid artery by disrupting local blood flow, a maneuver that causes permanent changes in shear stress conditions. One important feature of this approach is that vascular endothelium is not removed, thus underlining the importance of substances that are specifically released by or targeted to VECs. In addition, availability of a murine model of vascular remodeling is essential to exploit the informative potential of genome manipulation, which is usually carried out in mice.

The aim of the present study was to explore the potential beneficial effects of HK gene delivery on NI formation caused by ligation of the common carotid artery. The effectiveness of gene therapy was tested in wild-type mice as well as in genetically manipulated mice lacking the B₂ receptor gene (B₂^{-/-} mice)^{22,23} or carrying an exogenous transgene encoding for the human B₂ receptor (HB₂ transgenic mice).²⁴

Methods

Preparation of Replication-Deficient Adenovirus Vector Carrying the HK Gene

An adenovirus vector containing the HK gene (Ad.CMV-cHK) was generated as previously described.¹⁸ The expression of HK cDNA is under the control of the cytomegalovirus (CMV) enhancer/promoter, followed by a bovine growth hormone poly(A) signal sequence. An adenovirus harboring the β -galactosidase gene under the control of the CMV enhancer/promoter (Ad.CMV-LacZ) was also prepared as described.²⁶

Animals

All procedures complied with the standards for care and use of animal subjects as stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md). Experiments were carried out in male mice (aged 2 to 3 months). Swiss mice were purchased from Charles River (Varese, Italy). B₂^{-/-} mice, kindly provided by Dr Fred Hess (Merck Laboratories, Rahway, NJ), were generated by gene targeting and homologous recombination on a J129Sv genetic background.²² Wild-type J129Sv mice (Jackson Laboratory, Bar Harbor, Maine) served as controls for B₂^{-/-} mice. HB₂ transgenic mice were developed on a c57/B16 background.²⁴ Wild-type c57/B16 mice (Charles River, Varese, Italy) served as controls for HB₂ transgenic mice.

Surgical Procedures and Animal Treatment

In Swiss mice anesthetized with 2,2,2-tribromoethanol (88 mmol/100 g body wt IP), the left common carotid artery was exposed and ligated with 6-0 silk just proximal the carotid bifurcation to disrupt blood flow. At the same occasion, the animals were injected via the left femoral vein with sterile saline (vehicle) or 1.8×10⁹ plaque-forming units (pfu) of Ad.CMV-cHK or Ad.CMV-LacZ. In sham-operated mice, the carotid artery was exposed but not ligated.

Additional Swiss mice, undergoing the same experimental protocol described above, were given 1 of the following compounds for 14 days: the B₂ antagonist D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]BK (icatibant, 1 μ mol/kg body wt per day IP, by osmotic minipumps), the B₁ antagonist des-Arg⁹-[Leu⁸]BK (DALBK, 50 nmol/kg body wt per day IP, by osmotic minipumps), or the inhibitor of NO synthase N^G-nitro-L-arginine methyl ester (L-NAME, 1.4 mmol/kg body wt per day in drinking water).

In a separate set of experiments, B₂^{-/-}, HB₂, and their respective wild-type control mice underwent carotid artery ligation and were

injected via the left femoral vein with sterile saline (vehicle) or 1.8×10⁹ pfu Ad.CMV-cHK or Ad.CMV-LacZ.

Fourteen days after surgery, mice were euthanized for morphometric analysis. Each group consisted of at least 7 mice.

Hemodynamic Measurements

Systolic blood pressure and heart rate of unanesthetized Swiss mice were measured by tail-cuff plethysmography (Visitech) under basal conditions and 3, 7, and 14 days after carotid ligation and injection of Ad.CMV-cHK (n=6) or Ad.CMV-LacZ (n=5).

Expression of HK

Evidence of successful infection by systemic Ad.CMV-cHK delivery was obtained by measuring HK mRNA levels in tissues and recombinant protein in plasma. Swiss mice (n=3) were injected with Ad.CMV-cHK or Ad.CMV-LacZ (1.8×10⁹ pfu IV). Three days later, blood was withdrawn from the hearts of anesthetized mice. Carotid artery, thoracic aorta, kidney, heart, and liver were isolated and removed for total RNA extraction by the Trizol method (BRL). Reverse transcription-polymerase chain reaction (RT-PCR) Southern blot analysis with the use of specific oligonucleotide probes for HK (5' primer, 5'-AAC ACA GCC CAG TTT GT-3'; 3' primer, 5'-CTT CAC ATA AGA CAG CA-3'; and internal probe, 5'-GACCTCAAAAATCCTGCC-3') was performed as previously described.¹⁷ Expression of mouse β -actin mRNA in tissues was used as an internal control. Circulating HK levels were determined by ELISA with use of an antibody that recognizes only the active moiety of HK.¹⁶

Measurements of HK, Kinin, cGMP, and cAMP Levels in Urine

Twenty-four-hour urine collections were obtained under basal conditions and 3, 7, and 14 days after intravenous injection of Ad.CMV-cHK or Ad.CMV-LacZ (n=6 mice for each treatment) from Swiss mice placed in metabolic cages. Urinary HK levels were determined by ELISA.¹⁶ Urinary levels of cGMP and cAMP were determined by an enzyme immunoassay (Biotrak). Urine samples for kinin measurements were collected in ethanol to prevent enzymatic degradation. Kinins were measured by radioimmunoassay (Phenix), after extraction with Sep-Pak C18 columns (Waters).

Morphometric Analysis

Fourteen days after carotid ligation or sham operation, the mice were anesthetized and perfusion-fixed at a constant pressure (100 mm Hg) via the left ventricle with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.3). The whole left common carotid artery was excised and placed for 24 hours in 4% paraformaldehyde. Vessels were then processed for paraffin embedding. Five serial sections (200 μ m apart) of 3- μ m thickness were cut, starting from 1 mm below the carotid bifurcation and proceeding to the aortic arch. Sections were stained with hematoxylin-eosin. Morphometric analysis was performed in a blind fashion by use of a dedicated software package (KS300, Zeiss). The areas enclosed by the external elastic lamina (EEL) and internal elastic lamina (IEL) and the length of the IEL and EEL were measured. IEL was taken as an index of inward remodeling; EEL, as a measure of vascular constriction. The area of the media was calculated by subtracting the area delimited by the IEL from the area delimited by the EEL. The total number of cells in the NI and media was evaluated at a magnification of ×1000 with use of a calibrated grid. Cellular density in the NI and media was calculated by dividing total cell count by the respective area. For each carotid artery, the values obtained from the 5 sections were averaged.

Statistical Analysis

Data are expressed as mean±SEM. Multivariate repeated measures ANOVA was performed to test for interaction between time and the grouping factor. In multiple comparisons among independent groups in which ANOVA and the F test indicated significant differences, the statistical value was determined according to the method of Bonferroni. Differences within and between groups were determined by

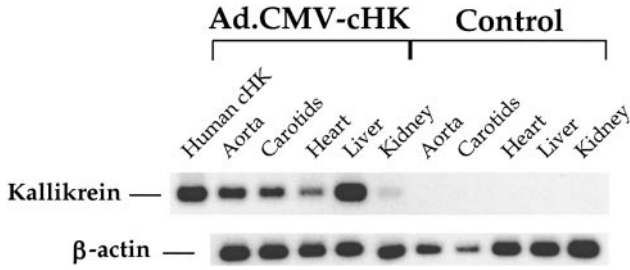


Figure 1. Top, HK mRNA in the mouse aorta, carotid artery, heart, liver, and kidney 3 days after intravenous injection of Ad.CMV-cHK (left) or Ad.CMV-LacZ (control, right). Bottom, Mouse β -actin mRNA levels. Human cHK indicates a positive control of HK cDNA.

paired or unpaired Student *t* test, respectively. A value of $P < 0.05$ was considered statistically significant.

Results

Expression of HK After Gene Delivery

The expression of HK mRNA in mice after kallikrein gene delivery was detected by RT-PCR, followed by Southern blot

analysis with the use of 3 oligonucleotides specific for HK. Figure 1 shows that HK mRNA can be detected in the aorta, carotid artery, heart, liver, and kidney but that the RT-PCR products from mice receiving Ad.CMV-LacZ did not hybridize to the HK gene probe (top panel). Similar levels of β -actin mRNA were detected in tissues of the experimental and control groups, verifying the quality of RNA in these samples (bottom panel). Three days after intravenous injection of Ad.CMV-cHK, circulating levels of immunoreactive HK averaged 145 ± 20 ng/mL. In addition, as shown in Figure 2A, immunoreactive HK was detected in the urine, peaking at 7 days after injection. In contrast, immunoreactive HK levels in plasma and urine were undetectable in mice injected with Ad.CMV-LacZ (see Figure 2).

Urinary Kinin, cGMP, and cAMP Levels After Gene Delivery

Urinary kinin, cAMP, and cGMP levels were not altered by Ad.CMV-LacZ (Figure 2B through 2D). In contrast, mice treated with Ad.CMV-cHK showed a 3.8-fold increase in

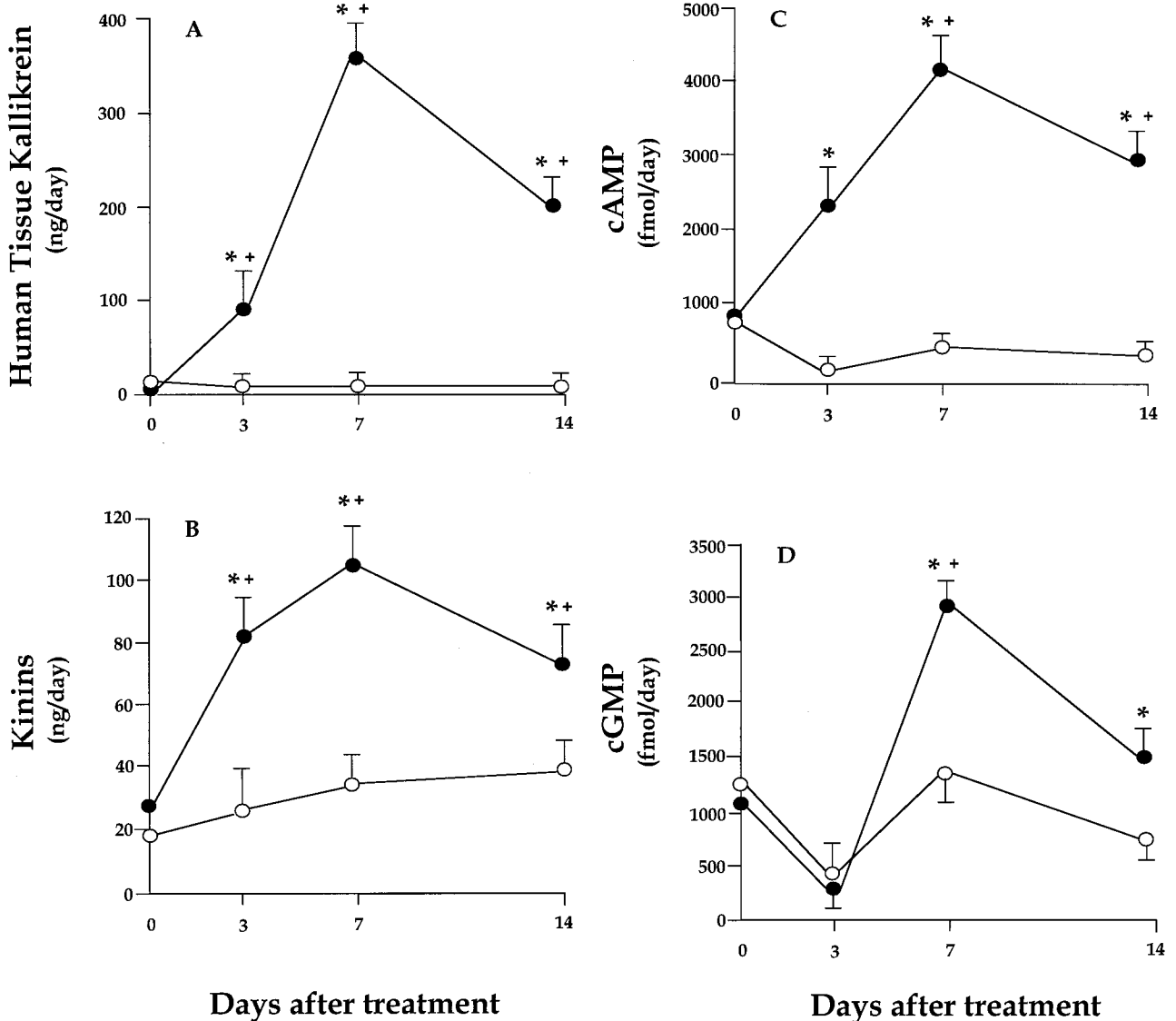


Figure 2. Urinary levels of HK (A), kinins (B), cAMP (C), and cGMP (D) under basal conditions (time 0) and at 3, 7, and 14 days after injection of 1.8×10^9 pfu of Ad.CMV-cHK (●, n=6) or Ad.CMV-LacZ (○, n=6) in the femoral vein of Swiss mice. * $P < 0.05$ vs Ad.CMV-LacZ; + $P < 0.05$ vs basal conditions.

immunoreactive kinin levels at 7 days after injection (Figure 2B). Urinary cAMP and cGMP increased by 5.3- and 2.5-fold, respectively (Figure 2C and 2D).

Morphometric Analysis

Consistent with previous studies performed in FVB mice,²⁰ we found that also in Swiss mice disruption of carotid blood flow causes a reduction in vessel lumen area that is due to a combination of NI formation (NI area $41\,817 \pm 5381 \mu\text{m}^2$), medial hyperplasia ($37\,445 \pm 5552$ versus $23\,820 \pm 5368 \mu\text{m}^2$ in sham-operated mice, $P < 0.05$), and reduction in IEL and EEL lengths (1095 ± 143 versus $1318 \pm 137 \mu\text{m}$ and 1162 ± 92 versus $1404 \pm 137 \mu\text{m}$, respectively; $P < 0.05$ for both comparisons). NI was maximally represented close to the ligation site and decreased in thickness in the direction of the aortic arch.

In Ad.CMV-LacZ-injected mice, the vascular remodeling response to carotid ligation was comparable qualitatively and quantitatively to that observed in mice given saline. In contrast, as shown in Figure 3, Ad.CMV-cHK delivery attenuated NI formation ($21\,059 \pm 3417$ versus $43\,885 \pm 2778 \mu\text{m}^2$ in Ad.CMV-LacZ-treated mice, $P < 0.05$) without affecting the medial area. As a consequence, the NI/media ratio was reduced by 52% (0.59 ± 0.18 versus 1.28 ± 0.06 in Ad.CMV-LacZ-treated mice, $P < 0.01$). The lengths of the IEL and EEL were unaffected by Ad.CMV-cHK delivery (972 ± 59 and $1064 \pm 72 \mu\text{m}$ versus 1071 ± 133 and $1158 \pm 111 \mu\text{m}$ in Ad.CMV-LacZ-treated mice, respectively; $P = \text{NS}$ for both comparisons).

Chronic administration of the B₁ or the B₂ receptor antagonists did not change the vascular remodeling response to carotid ligation in Ad.CMV-LacZ-treated mice (data not shown). However, when these agents were tested in Ad.CMV-cHK-treated mice, we found that the B₂ antagonist icatibant completely abrogated the protective effect of HK gene delivery against NI formation, whereas the B₁ receptor antagonist DALBK was ineffective (Figure 3).

As shown in Figure 4, HK gene transfer decreased NI total cell count (101 ± 20 versus 268 ± 65 cells in the cross section Ad.CMV-LacZ group, $P < 0.01$) and increased NI cell density (22 ± 2 versus 6 ± 1 cells/mm² in the Ad.CMV-LacZ group, $P < 0.001$). In contrast, HK gene delivery produced borderline changes in medial total cell count (149 ± 14 versus 109 ± 10 cells cross section in the Ad.CMV-LacZ group, $P = 0.06$) and in medial cell density (7 ± 1 versus 5 ± 1 cells/mm² in the Ad.CMV-LacZ group, $P = 0.09$). Icatibant contrasted the effect of kallikrein on NI cell count and density (Figure 4A) without affecting these parameters in the media (Figure 4B). The B₁ antagonist DALBK did not alter the effects of HK gene delivery on cellular count and density.

Figure 5 shows the typical pattern of vascular remodeling caused by ligation of the carotid artery in Swiss mice treated with Ad.CMV-LacZ (panel B), Ad.CMV-cHK (panel C), or Ad.CMV-cHK plus icatibant (panel D).

In Ad.CMV-LacZ-treated Swiss mice, L-NAME did not alter the remodeling response to carotid ligation. Evaluation of the effect of NO synthase inhibition after HK gene transfer was precluded by a dramatic reduction in the survival rate in the group given L-NAME in combination with Ad.CMV-cHK. In fact, all 12 mice that entered this treatment showed

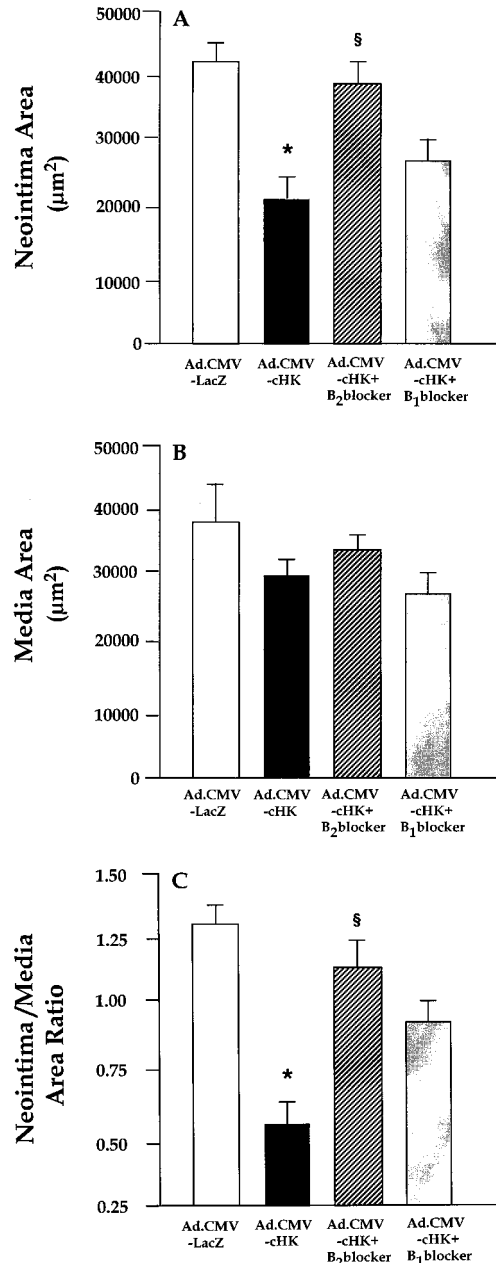


Figure 3. NI area (A), medial area (B), and ratio between NI and medial areas (C) 14 days after disruption of blood flow in the ligated carotid of Swiss mice given intravenous injections of Ad.CMV-LacZ (open bars, n=13), Ad.CMV-cHK (solid bars, n=15), Ad.CMV-cHK in combination with the kinin B₂ receptor blocker icatibant ($1 \mu\text{mol/kg}$ body wt per day IP, by osmotic minipump; hatched bars, n=8), or Ad.CMV-cHK plus the B₁ blocker DALBK (50 nmol/kg body wt per day IP, by osmotic minipump; shaded bars, n=8). * $P < 0.05$ vs Ad.CMV-LacZ; § $P < 0.05$ vs Ad.CMV-cHK.

a progressive deterioration of general conditions and died within 7 days.

Effect of Ad.CMV-cHK Delivery on Vascular Remodeling in B₂^{-/-} and HB₂ Transgenic Mice

Morphometric analysis of carotid arteries did not detect any difference between B₂^{-/-} and HB₂ transgenic mice and their respective wild-type controls, J129Sv and c57/B16, in the absence of vascular injury as well as after artery ligation (data not shown). However, genetically manipulated animals dif-

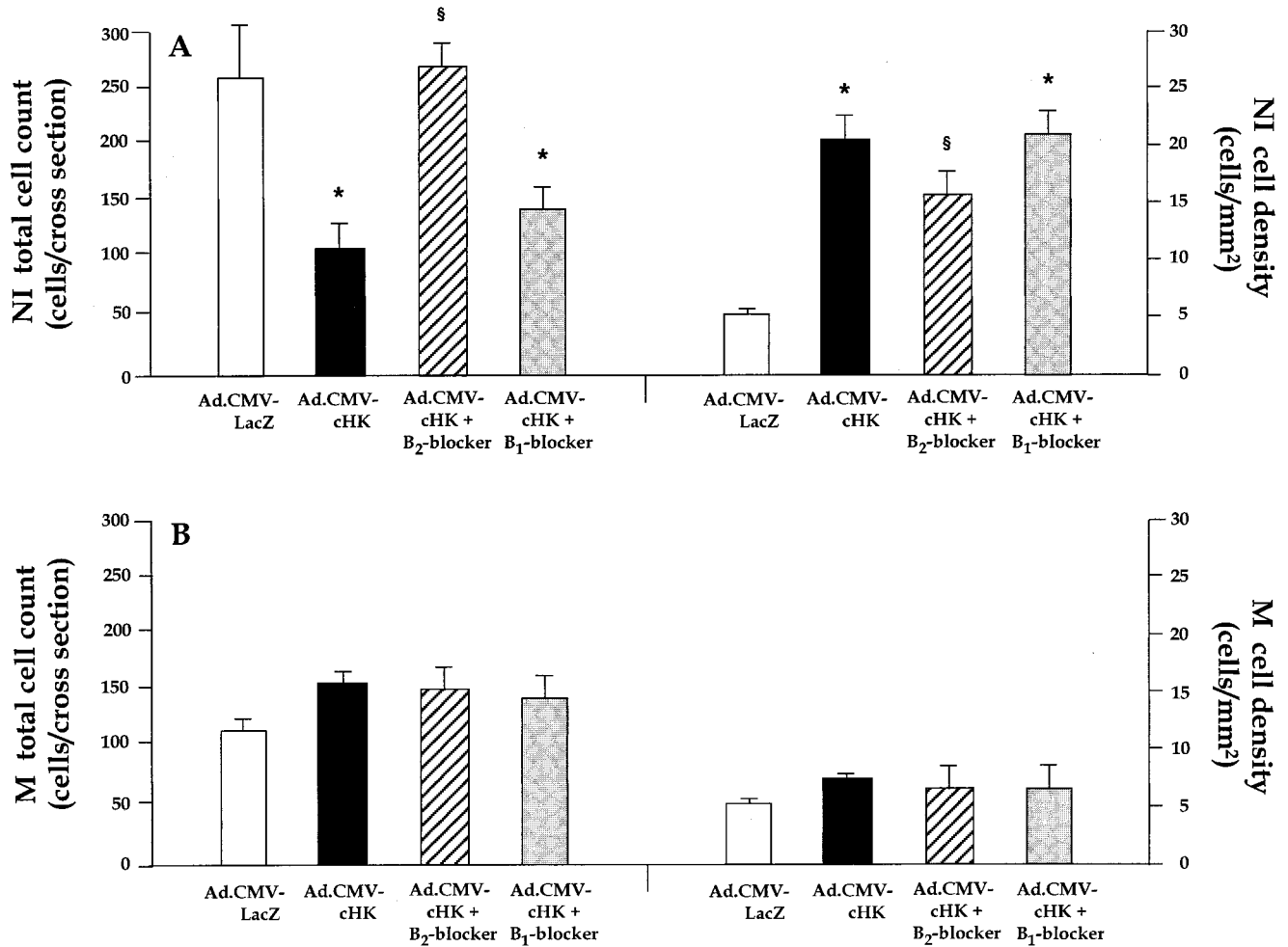


Figure 4. Total cell count per cross section and cell density in NI (A) and media (M, B) 14 days after disruption of blood flow in the ligated carotid arteries of Swiss mice given intravenous injections of Ad.CMV-LacZ (open bars, $n=13$), Ad.CMV-cHK (solid bars, $n=15$), Ad.CMV-cHK plus icatibant (hatched bars, $n=8$), or Ad.CMV-cHK plus DALBK (shaded bars, $n=8$). * $P<0.05$ vs Ad.CMV-LacZ; $§P<0.05$ vs Ad.CMV-cHK.

ferred from controls regarding the effectiveness of HK in preventing NI formation. In fact, as shown in Figure 6A (left bars), the protective effect of Ad.CMV-cHK delivery was reduced in the $B_2^{-/-}$ strain compared with the wild-type J129Sv strain, leading to a greater NI/media area ratio in knockout mice (Figure 6C, left bars). Conversely, inhibition of NI formation by Ad.CMV-cHK was potentiated in HB₂ transgenic mice compared with their wild-type controls, c57/Bl6 (Figure 6, right bars).

Hemodynamic Measurements

As shown in the Table, no change in systolic blood pressure was observed in Swiss mice after ligation of the common carotid artery and injection of Ad.CMV-cHK or Ad.CMV-LacZ. A tendency of the heart rate to increase was observed in both groups, but this change did not reach statistical significance ($P=0.08$).

Discussion

To the best of our knowledge, this is the first study demonstrating a protective role of adenovirus-mediated HK gene delivery against vascular remodeling in the mouse. Another novel discovery is that insertion or deletion of the BK B_2 receptor gene respectively enhances or precludes the protec-

tive action of the tissue kallikrein gene on NI formation, thus demonstrating the essential role of B_2 receptor signaling in this gene therapy approach.

Injury to the arterial wall induces the synthesis of gene products that stimulate smooth muscle cell migration and proliferation, thus leading to intimal hyperplasia. It has been hypothesized that the suppression of tissue kallikrein gene expression in the damaged vessel may contribute to the pathogenesis of restenosis after angioplasty.¹⁹ This idea is supported by the observation that kinins, generated from kininogen by tissue kallikrein, inhibit vascular cell proliferation.¹⁹ On the basis of this assumption, HK gene delivery has been successfully used to suppress NI formation in the rat balloon-injured carotid artery, an effect that appears to be mediated by activation of the kinin B_2 receptor.

A large body of evidence now indicates that a chronic decrease in shear stress can also produce vascular wall alterations similar to those caused by mechanical injury in normal and in atherosclerotic vessels.²⁷⁻²⁹ Therefore, we thought it would be worthwhile to evaluate the efficacy of HK gene delivery in preventing the vascular remodeling caused by permanent alteration in shear stress conditions. To this aim, we exploited the mouse model recently established by Kumar and Lindner,²⁰ in which disruption

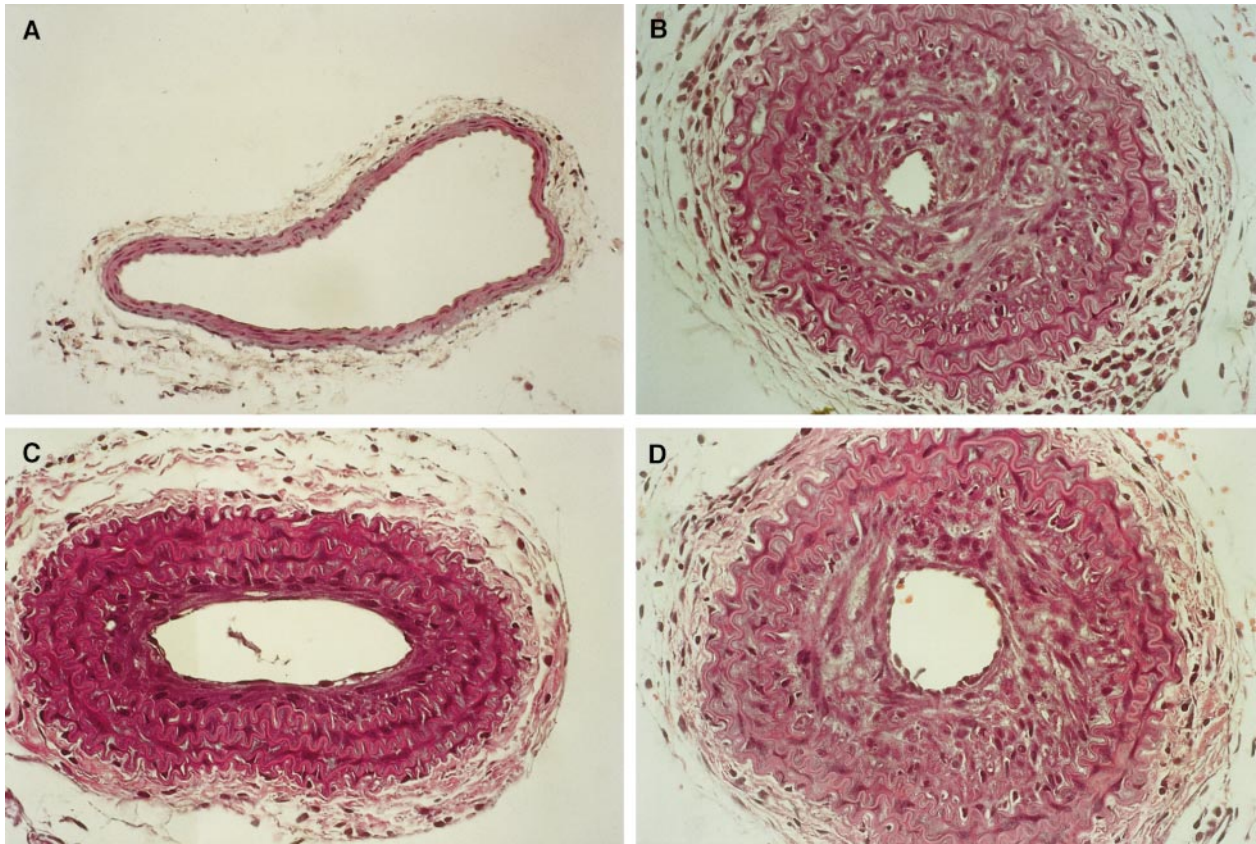


Figure 5. Representative hematoxylin-eosin-stained carotid transverse sections taken 1 mm apart from the carotid bifurcation of sham-operated Swiss mice (A, magnification $\times 100$) or ligated left carotid arteries of Swiss mice treated with Ad.CMV-LacZ (B, magnification $\times 400$), Ad.CMV-cHK alone (C, magnification $\times 400$), or Ad.CMV-cHK in combination with icatibant (D, magnification $\times 400$).

of blood flow triggers NI formation together with reduction in vessel diameter. In this setting, the proliferative response of VSMCs is thought to be stimulated by the increase in arterial wall tension that occurs proximal to the ligation.^{27,28}

Under basal conditions, we found no difference between $B_2^{-/-}$ and wild-type control mice regarding the vascular structure of the carotid artery; neither B_2 receptor gene knockout altered vascular remodeling after ligation. Thus, the congenital absence of the B_2 receptor neither results in structural vascular abnormalities nor affects the remodeling response to vascular injury. Similarly, NI formation was not worsened by B_2 receptor antagonism in Swiss mice injected with Ad.CMV-LacZ. However, a functional B_2 receptor appears to be essential for the vascular protection exerted by HK gene delivery. In fact, the suppression of NI formation by Ad.CMV-cHK was abrogated by the B_2 receptor antagonist icatibant. These data are in line with the results obtained by Farhy et al¹⁵ in the rat balloon-injury model by the use of an ACE inhibitor. In fact, icatibant, which by itself did not worsen NI formation, partially prevented the protective effect of ACE inhibition. Altogether, these results indicate that kinins are indeed able to contrast vascular remodeling, provided that their levels are augmented through pharmacological or genetic interventions.

This assumption, together with the identification of the receptor implicated in the action of Ad.CMV-cHK, was further challenged by the use of murine models in which the endogenous B_2 receptor gene was either deleted or added to

the human B_2 receptor gene. This approach has obvious advantages over the classic use of receptor antagonists, including the possibility of recognizing a correlation between biological effects of HK and the number of copies of the B_2 receptor gene. A gene dosage effect was found. In fact, we have shown that the protective action of Ad.CMV-cHK is virtually absent in $B_2^{-/-}$ and enhanced in HB_2 transgenic mice, thus demonstrating the essential role of the B_2 receptor in the beneficial effect of HK gene delivery.

The HK gene was expressed in the vasculature, liver, kidney, and heart after intravenous injection of Ad.CMV-cHK into mice. The secreted nature of the gene product is indicated by the presence of active HK in the circulation and urine. Therefore, suppression of NI formation by Ad.CMV-cHK may be attributable to the effect of recombinant protein either locally expressed or circulating in the blood stream. The protection exerted by Ad.CMV-cHK delivery appears to be limited to a reduction in NI formation, whereas medial hyperplasia or vessel shrinking remained unaffected. The finding that after Ad.CMV-cHK treatment total cell count was decreased in the NI and unchanged in the media suggests that HK could act by inhibiting VSMC proliferation. An antiproliferative role of Ad.CMV-cHK on cultured VSMCs has been previously shown by Murakami et al.¹⁹ In vitro experiments also indicate that the binding of kinins to aortic VSMC receptors stimulates PGI_2 formation, with increased cAMP levels and subsequent inhibition of VSMC proliferation.³⁰ The mechanism by which Ad.CMV-cHK transfer inhibits vascular growth in vivo may also involve the induction and/or

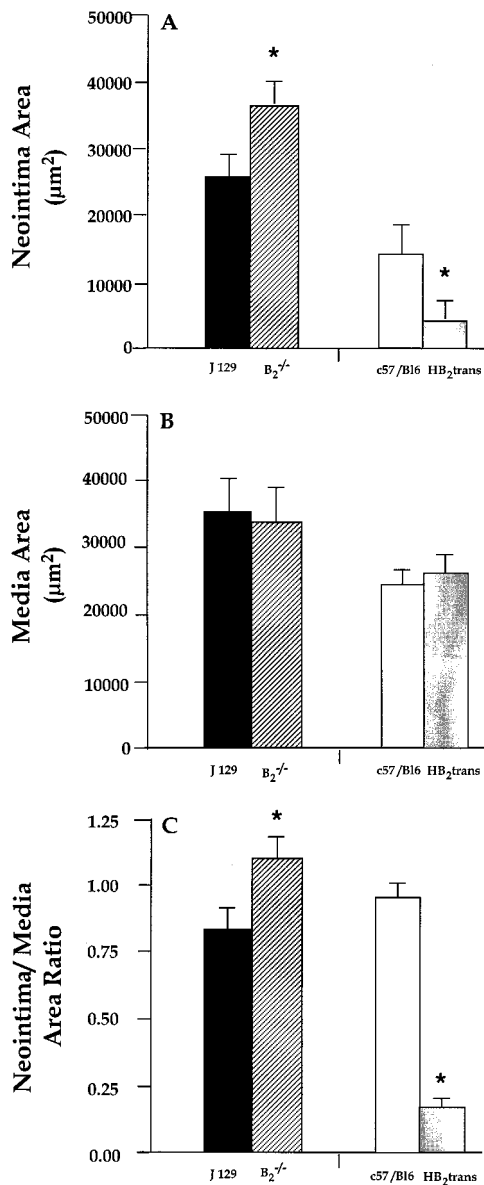


Figure 6. Effects of systemic delivery of Ad.CMV-cHK in carotid-ligated kinin B₂ receptor gene knockout mice (B₂^{-/-}, n=8; hatched bars) and their wild-type controls (J129, n=8; solid bars) and in transgenic mice harboring the human B₂ receptor (HB₂trans, n=7; shaded bars) and their wild-type controls (c57/Bl6, n=9; open bars). NI area (A), medial area (B), and ratio between NI and medial areas (C) are plotted. *P<0.05 vs respective wild-type controls.

activation of NO synthase. The binding of kinins to vascular endothelial B₂ receptor increases NO release from VECs.³¹ Once released by VECs, NO reaches VSMCs, where it activates guanylate cyclase, thus increasing the production of

cGMP, a potent inhibitor of VSMC proliferation and migration.^{7,8,32} Accordingly, previous studies showed that kinin-stimulated NO release contributes to the beneficial effect of ACE inhibitors in balloon injury-induced remodeling by reducing VSMC migration from the tunica media to the NI.^{15,33} As stated above, cell count in the media was unchanged by Ad.CMV-cHK treatment. We would expect that if HK blocked VSMC migration to NI without affecting the proliferation rate, the total number of cells would be increased in the tunica media. Because this was not the case, it is likely that VSMC migration and proliferation were inhibited by Ad.CMV-cHK treatment. Further studies that use markers of cellular turnover may help dissect the importance of these 2 mechanisms. The finding that NI cell density was augmented in the Ad.CMV-HK group favors a suppressive role of HK against matrix accumulation.

Although activation of cAMP and cGMP pathways by HK is well documented in the present study, investigation of the role of NO was precluded by the fact that L-NAME dramatically reduced the survival rate in mice given the HK gene, possibly because of severe bronchoconstriction and hypertension.^{34,35} L-NAME by itself did not affect remodeling in animals treated with Ad.CMV-LacZ. This is in apparent discordance with studies of vascular remodeling in endothelial NO synthase knockout mice, which show increased wall thickness after carotid ligation, compared with wild-type control mice.⁹ However, it should be noted that only the external carotid artery was ligated in endothelial NO synthase knockout mice,⁹ whereas in our experimental setting, blood flow was completely interrupted by ligation of the common carotid artery. Abrogation of blood flow may have reduced the basal release of NO from vascular endothelium to such an extent that no further decrease would be expected with NO synthase inhibition.

Although carotid occlusion could disturb blood flow to the brain and baroreceptor function, this had no consequence on systemic blood pressure. Failure to detect changes in blood pressure could be due to the fact that adaptive adjustments may have already occurred at the time the first measurements were performed. In addition, because of an efficient collateral flow, ligation of 1 carotid artery may be not sufficient to induce brain ischemia in mice. Disturbance in baroreceptor function is supported by the tachycardia observed after surgery.

In conclusion, we have demonstrated the feasibility and efficacy of adenovirus-mediated HK gene transfer in a mouse model of vascular remodeling. These results underline the importance of the kallikrein-kinin system in vascular biology and open a new avenue for gene therapy in vascular diseases.

SBP and HR Measured Under Basal Conditions and at Different Time Points After Injection of 1.8×10⁹ pfu Ad.CMV-cHK or Ad.CMV-LacZ in Femoral Veins of Swiss Mice

Groups	After Injection							
	Basal		3 d		7 d		14 d	
	SBP, mm Hg	HR, bpm	SBP, mm Hg	HR, bpm	SBP, mm Hg	HR, bpm	SBP, mm Hg	HR, bpm
HK (n=6)	116±5	571±32	112±4	640±32	116±3	587±29	124±5	676±34
LacZ (n=5)	118±2	475±13	121±4	580±19	124±2	640±25	119±2	587±37

Values are mean±SEM. SBP indicates systolic blood pressure; HR, heart rate; HK, Ad.CMV-cHK-injected group; and LacZ, Ad.CMV-LacZ-injected group.

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References

- Guyton JR, Hartley CJ. Flow restriction of one carotid artery in juvenile rats inhibits growth of arterial diameter. *Am J Physiol.* 1985;248:H540-H546.
- Langille BL, O'Donnell F. Reductions in arterial diameter produced by chronic decreases in flow are endothelium-dependent. *Science.* 1986;231:405-407.
- Kamiya A, Togawa T. Adaptive regulation of wall shear stress to flow change in the canine carotid artery. *Am J Physiol.* 1980;293:H14-H21.
- Nolly H, Carretero OA, Scicli G, Madeddu P, Scicli G. A kallikrein-like enzyme in blood vessels of one-kidney, one-clip hypertensive rat. *Hypertension.* 1990;16:436-440.
- Madeddu P, Varoni MV, Demontis MP, Fattaccio MC, Parpaglia PP, Glorioso N. A kallikrein-like enzyme in the aorta of normotensive and hypertensive rats. *Hypertension.* 1994;23:899-902.
- Madeddu P, Gherli T, Bacciu PP, Maioli M, Glorioso N. A kallikrein-like enzyme in human vascular tissue. *Am J Hypertens.* 1993;6:344-348.
- Grag UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromocyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. *J Clin Invest.* 1989;83:1774-1777.
- Sarkar R, Meinberg EG, Stanley JC, Gordon D, Webb RC. Nitric oxide reversibly inhibits the migration of cultured vascular smooth muscle cells. *Circ Res.* 1996;78:225-230.
- Rudic RD, Shesley EG, Maeda N, Smithies O, Segal S, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest.* 1998;101:731-736.
- Graves LM, Bornfeldt KE, Raines EW, Potts BC, McDonald SG, Ross R, Krebs EG. Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. *Proc Natl Acad Sci U S A.* 1993;90:10300-10304.
- Kato J, Matsuoka M, Polyak K, Massague J, Sherr CJ. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27^{kip1}) of cyclin-dependent kinase 4 activation. *Cell.* 1994;79:487-496.
- Powell JS, Clozel JP, Muller RKM, Kuhn H, Hefti F, Hosang M, Baumgartner HR. Inhibitors of angiotensin-converting enzyme prevent myointimal proliferation after vascular injury. *Science.* 1989;245:186-188.
- Taguchi J, Abe J, Okazaki H, Ochiai M, Ohno M, Takuwa Y, Kurokawa K. Angiotensin converting enzyme inhibitors or Dup753 prevent neointimal formation following balloon injury with single topical or multiple systemic application. *Biochem Biophys Res Commun.* 1993;196:969-974.
- deBlois D, Lombardi DM, Garvin MA, Schwartz SM. Inhibition by ramipril of intimal hyperplasia in the denuded rat carotid is reversed by Hoe 140, a kinin B₂ receptor antagonist. *Circulation.* 1992;86(suppl I):I-226. Abstract.
- Farhy DR, Carretero OA, Ho KL, Scicli AG. Role of kinins and nitric oxide in the effects of ACE inhibition on neointima formation. *Circ Res.* 1993;72:1202-1210.
- Jin L, Zhang J, Chao L, Chao J. Gene therapy in hypertension: adenovirus-mediated kallikrein gene therapy in hypertensive rats. *Hum Gene Ther.* 1997;8:1753-1761.
- Chao J, Zhang J, Lin KF, Chao L. Adenovirus-mediated kallikrein gene delivery attenuates hypertension, cardiac hypertension and renal injury in Dahl-salt sensitive rats. *Hum Gene Ther.* 1998;9:21-31.
- Yayama K, Wang C, Chao L, Chao J. Kallikrein gene delivery attenuates hypertension, cardiac hypertrophy and enhances renal function in Goldblatt hypertensive rats. *Hypertension.* 1998;31:1104-1110.
- Murakami H, Yayama K, Miao RQ, Wang C, Chao L, Chao J. Kallikrein gene delivery inhibits vascular smooth muscle cell growth and neointima formation in rat artery after balloon angioplasty. *Hypertension.* 1999;34:164-170.
- Kumar A, Lindner V. Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol.* 1997;17:2238-2244.
- Kumar A, Hoover JL, Simmons CA, Lindner V, Shebuski RJ. Remodeling with neointima formation in the carotid artery of normal and P-selectin-deficient mice. *Circulation.* 1997;96:4333-4342.
- Borkowski JA, Ranson RW, Seabrook GR, Trumbauer M, Chen H, Hill RG, Strader CD, Hess JF. Targeted disruption of bradykinin B₂ receptor gene in mice eliminates bradykinin action in smooth muscle and neurons. *J Biol Chem.* 1995;270:13706-13710.
- Madeddu P, Varoni MV, Palomba D, Emanuelli C, Demontis MP, Glorioso N, Dessi Fulgheri P, Sarzani R, Anania V. Cardiovascular phenotype of a mouse strain with disruption of bradykinin B₂ receptor gene. *Circulation.* 1997;96:3570-3578.
- Wang DZ, Chao L, Chao J. Hypotension in transgenic mice overexpressing human bradykinin B₂ receptor. *Hypertension.* 1997;29:488-493.
- Deleted in proof.
- Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Alam T, Gerard RD, Newgard CB. Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol.* 1994;43:161-189.
- Langille BL, Bendeck MP, Keely FW. Adaptation of carotid arteries of young and mature rabbits to reduced carotid blood flow. *Am J Physiol.* 1989;256:H931-H939.
- Glasgow S. Intimal hyperplasia, vascular remodeling, and the restenosis model. *Circulation.* 1994;89:2888-2891.
- Kohler TR, Jawen A. Flow affects development of intimal hyperplasia after arterial injury in rats. *Arterioscler Thromb.* 1992;12:963-971.
- Dixon BS, Breckon R, Fortune J, Vavrek RJ, Stewart JM, Marzec-Calvert R, Linas SL. Effects of kinins on cultured arterial smooth muscle. *Am J Physiol.* 1990;258:C299-C308.
- Zhang X, Scicli G, Xu A, Nasjletti A, Hintze TH. Role of endothelial kinins in control of coronary nitric oxide production. *Hypertension.* 1997;30:1105-1111.
- Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol.* 1994;276:C1405-C1413.
- Farhy DR, Peterson E, Scicli AG. Kinins and the events influenced by an angiotensin-converting enzyme inhibitor during neointima formation in the rat carotid artery. *J Hypertens.* 1997;15:421-429.
- Figini M, Ricciardolo FL, Javdan P, Nijkamp FP, Emanuelli C, Pradelles P, Folkerts G, Geppetti P. Evidence that epithelium-derived relaxing factor released by bradykinin in the guinea pig trachea is nitric oxide. *Am J Respir Crit Care Med.* 1996;153:918-923.
- Ricciardolo FL, Di Maria GU, Mistretta A, Sapienza MA, Geppetti P. Randomised double-blind placebo-controlled study of the effect of inhibition of nitric oxide synthesis in bradykinin-induced asthma. *Lancet.* 1996;348:374-377.