

Targeting Kinin B₁ Receptor for Therapeutic Neovascularization

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Background—Kinins are modulators of cardiovascular function. After ischemic injury, enhanced kinin generation may contribute in processes responsible for tissue healing.

Methods and Results—Using pharmacological and genetic approaches, we investigated the role of kinin B₁ receptor in reparative angiogenesis in a murine model of limb ischemia. The effect of B₁ pharmacological manipulation on human endothelial cell proliferation and apoptosis was also studied in vitro. Abrogation of B₁ signaling dramatically inhibited the native angiogenic response to ischemia, severely compromising blood perfusion recovery. Outcome was especially impaired in B₁ knockouts that showed a very high incidence of limb necrosis, eventually leading to spontaneous auto-amputation. Conversely, local delivery of a long-acting B₁ receptor agonist enhanced collateral vascular growth in ischemic skeletal muscle, accelerated the rate of perfusion recovery, and improved limb salvage. In vitro, B₁ activation stimulated endothelial cell proliferation and survival, whereas B₁ antagonism induced apoptosis.

Conclusions—Our results indicate that the B₁ plays an essential role in the host defense response to ischemic injury. B₁ signaling potentiation might be envisaged as a utilitarian target for the treatment of ischemic vascular disease. (*Circulation*. 2002;105:360-366.)

Key Words: receptors, bradykinin ■ angiogenesis ■ ischemia ■ muscle, skeletal ■ endothelium

Ischemic disease represents a major health problem and is expected to become the new worldwide epidemic of the third millennium. After occurrence of vascular occlusion, clinical outcome mainly depends on the native potential to develop new collaterals, a defense response aimed to maintain tissue perfusion and function. However, under certain conditions such as diabetes, hypercholesterolemia, and hypertension, post-ischemic healing is compromised because of a defective modulation of angiogenic growth factors.¹⁻³ Thus, supplementation with angiogenic substances has been proposed as a possible mechanism to overcome the failure in mounting an adequate vascular response.^{4,5}

The kallikrein-kinin system (KKS), long regarded as a modulator of cardiovascular homeostasis, nociception, and inflammation,^{6,7} is now being considered for its potential in reparative and therapeutic angiogenesis.^{3,8,9} Kinin peptides cleaved from kininogens by kallikreins interact with G protein-coupled B₁ and B₂ receptors (B₁ and B₂), thus leading vascular endothelial cells (ECs) to release autacoids such as nitric oxide (NO) and prostaglandins.^{10,11} The constitutive B₂ is responsible for the majority of biological effects of brady-

kinin (BK) and Lys-BK. In contrast, the B₁ preferentially binds desArg⁹-BK and Lys-desArg¹⁰-BK, is induced by tissue damage via activation of transcription factor nuclear factor κ B, and does not desensitize after exposure to ligand.¹²⁻¹⁴ Circumstantial evidence for a role of B₁ in reparative angiogenesis comes from the observation that B₁ gene expression is up-regulated by ischemia in skeletal muscle or myocardium.^{8,15} In vitro, kinins promote EC proliferation via activation of the B₁-cAMP pathway.^{16,17} In vivo, B₁ stimulation induces neovascularization in the rabbit cornea¹⁷ and is responsible for capillary proliferation evoked by BK¹⁸ or tissue kallikrein gene transfer.^{8,19}

The aim of the present study was to firmly establish the functional relevance of B₁ in post-ischemic neovascularization. We demonstrated that B₁ signaling plays an essential role in reparative angiogenesis by modulating EC proliferation and survival. Moreover, potentiation of this mechanism exerts a therapeutic effect by accelerating spontaneous healing. Exploitation of the angiogenic potential of B₁ agonists may be envisaged for the treatment of patients with peripheral artery disease.

Received July 12, 2001; revision received October 31, 2001; accepted November 2, 2001.

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Methods

All procedures complied with the standards stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md, 1996). CD1 mice were purchased from Charles River, Comerio, Italy. B₁ knock outs²⁰ were provided by Max Delbrück Center for Molecular Medicine (Berlin-Buch, Germany), whereas their wild-type J129 controls were obtained from Jackson Laboratories (Bar Harbor, Me).

In Vivo Experimental Protocols

With mice under the effect of 2,2,2 tribromoethanol anesthesia (880 mmol/kg BW IP, Sigma-Aldrich), the left femoral artery was exposed, dissected free, and excised.⁸ Three groups were studied: (1) CD1 mice were given the B₁ antagonists desArg⁹-[Leu⁸]-BK (DALBK from Sigma-Aldrich, at 50 nmol/kg BW per day, n=9) or AcLys[D-βNal⁷,Ile⁸]-desArg⁹-BK (R715, at 50 nmol/kg BW per day, n=7), or vehicle (sterile saline, n=8), and were followed-up for 21 days from surgery. Drugs were delivered IP by osmotic mini-pumps (Alza Co., Palo Alto, CA). Selectivity of antagonists has been previously reported.⁷ (2) The second group consisted of B₁ knock-outs (n=15) and J129 controls. The latter were subdivided into 2 groups given DALBK (n=8) or saline (n=10) IP. Mice were euthanized 14 days from surgery. (3) CD1 and J129 mice had their left adductor muscles injected with B₁ agonist Sar[D-Phe⁸]-des-Arg⁹-BK (R916, at 2.5 μmol/kg BW in 20 μL saline once or twice a day, starting on the day of surgery, n=10 each dose per strain) or vehicle (n=10 each strain) and were followed-up for 14 days from ischemia.

Histological Assessment of Neovascularization

Capillary and myofiber densities of both adductors were determined at necropsy. Anesthetized mice were perfused with PBS (1 minute) and then with 10% buffered formalin (10 minutes) at 100 mm Hg via the abdominal aorta. After paraffin embedding, 3 μm-thick sections were cut from each sample with muscle fibers transversely oriented, and stained with hematoxylin and eosin.

Sections were analyzed in a blinded fashion using an ocular reticle (9604-μm² area) at 1000× magnification. Twenty-five fields were randomly counted and averaged. The number of capillary (n_{cap}) and myofiber (n_{fiber}) profiles per field was used to compute capillary or myofiber numerical density per square mm of muscle, according to the following formulas: n_{cap}/mm² = n_{cap} in total fields/total field area and n_{fiber}/mm² = n_{fiber} in total fields/total field area. In addition, capillary density was normalized to myofiber density (n_{cap}/n_{fiber}).^{3,8}

Post-Ischemic Hemodynamic Recovery

Hindlimb blood flow of anesthetized mice was measured by laser Doppler flowmetry (Lisca, Sweden) under basal conditions and then weekly after surgery, and the ratio of perfusion between ischemic to non-ischemic foot was calculated.⁸

EC Proliferation and Apoptosis

Human umbilical vein ECs (HUVECs) were cultured in M199 supplemented with 20% fetal bovine serum (FBS, Gibco), 0.3% bovine brain extract (BBE, Clonetics), and antibiotics, and were maintained at 37°C in 5% CO₂/95% air. Experiments were performed on cells at passages between 2 and 5.

The effect of B₁ stimulation on HUVEC proliferation was assayed after a total of 24 hours of serum starvation. In a set of experiments, the last 2 hours of starvation were combined with incubation in a modified Krebs buffer (137 mmol/L NaCl, 3.8 mmol/L KCl, 0.49 mmol/L MgCl₂, 0.9 mmol/L CaCl₂, and 4.0 mmol/L Hepes) supplemented with 10 mmol/L 2-deoxyglucose, 20 mmol/L sodium lactate, 1 mmol/L sodium dithionite, and 12 mmol/L KCl (pH 6.5) to evoke simulated ischemia (SI).²¹ Then, ischemic or not ischemic HUVECs were incubated for 72 hours in M199 medium containing 1% FCS and added with the B₁ agonist R916 (50 nmol/L to 1 μmol/L) or vehicle (PBS). In a separate experiment, R916 (100 nmol/L) was co-incubated with either R715 (1 μmol/L) or PBS. Incubation was carried out in the presence of 1 μCi/mL [³H]

thymidine (specific activity 5 Ci/mmol, Amersham Pharmacia Biotech). The medium was then removed and the cell monolayer in each well was washed twice with PBS, exposed to 5% trichloroacetic acid (500 μL) for 5 minutes, and fixed in methanol (500 μL). Finally, cells were digested by adding 25 mol/L formic acid (500 μL). Each digest was transferred to a scintillation vial and radioactivity was determined by a scintillation counter (LKB Instrument Inc). Experiments were repeated at least 6 times.

To assess the effect of B₁ blockade on apoptosis, HUVECs suspended in a normal culture medium were seeded in gelatinated 24-well dishes at a density of 4×10⁴ cells per well, and allowed to attach overnight. Fresh media supplemented with low (2%) or normal (20%) FBS and containing R916 (100 nmol/L to 5 μmol/L), R715 (100 nmol/L to 5 μmol/L), or PBS was then added for 24 or 48 hours. Experiments were performed at the standard atmosphere or under hypoxia obtained by placing cell plates in a modular incubator chamber (Billups-Rothenberg) filled with 5% CO₂/95% N₂. At the end of the incubation period, supernatant was collected for LDH determination by the Promega Cytotoxic Assay and apoptosis was investigated by 4'-6'-diaminidino phenylindole (DAPI) staining (Roche). To this aim, cells were fixed in 4% paraformaldehyde for 10 minutes, washed twice with PBS and incubated with DAPI (1 μg/mL PBS) for 20 minutes. Wells were examined by fluorescent microscopy at 100× magnifications. Three randomly selected fields per well were photographed with a videocamera and then analyzed for total cell number. Apoptotic HUVECs, identified by condensation and fragmentation of their nuclei, were counted by a researcher blinded to the treatment. In each examined field, the total cell number was also computed to calculate the percent of apoptotic cells. Each experiment was repeated 3 to 6 times.

Statistical Analysis

All results are expressed as mean±SEM. 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 *F* test indicated significant differences, the statistical value was determined according to the Bonferroni's method. Differences within and between groups were determined using paired or unpaired Student's *t* test, respectively. The comparative incidence of limb salvage was evaluated by χ² analysis. A *P* value <0.05 was interpreted to denote statistical significance.

Results

Impact of B₁ Signaling on Microvascular Response to Ischemia

As depicted in Figure 1A, vehicle-treated J129 showed a 1.7-fold increase in adductor muscle capillarity 14 days from ischemia induction (950±158 versus 544±66 cap/mm² in contralateral normoperfused muscles, *P*<0.01). The reparative response was attenuated by DALBK (773±71 versus 603±92 cap/mm² in contralateral muscles, *P*=NS) and absent in B₁ knockouts (367±59 versus 383±45 cap/mm² in contralateral muscles, *P*=NS). Results did not differ when expressed as capillary to myofiber ratio (Figure 1B).

As shown in Figure 2, the instrumental relevance of endogenous B₁ signaling was confirmed in the CD1 strain. In fact, although capillary density was increased by 1.9-fold 3 weeks from induction of ischemia in vehicle-treated animals (1008±137 versus 516±79 cap/mm² in contralateral adductors, *P*<0.001), this response was prevented by either DALBK or R715 (*P*<0.01 for both comparisons). Similar results were obtained after normalization of capillarity by fiber number (data not shown).

We then evaluated whether reparative neovascularization would be potentiated by R916 agonist local administration.

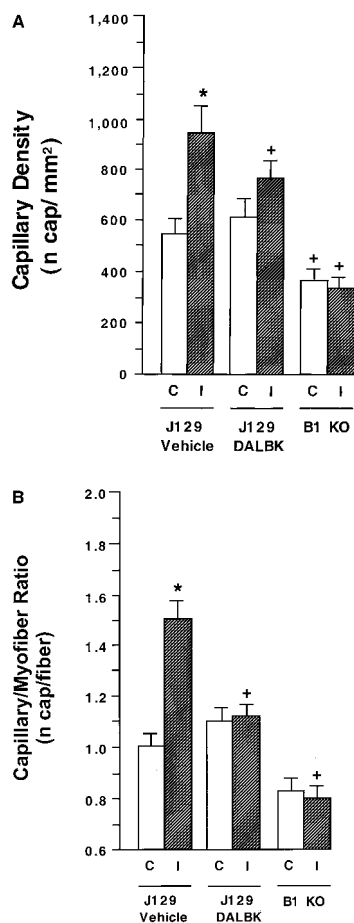


Figure 1. Bar graph shows the effect of B_1 receptor signaling abrogation on ischemia-induced neovascularization. Fourteen days from femoral artery excision, vehicle-treated wild-type J129 showed higher capillary density (panel A) and capillary/myofiber ratio (panel B) in ischemic adductor muscles (I, hatched columns) than in contralateral normoperfused muscles (C, open columns). The response to ischemia was blunted by the B_1 antagonist DALBK in J129 and absent in B_1 knockouts (B_1 KO). Values are mean \pm SEM. * $P < 0.05$ vs contralateral, $^+P < 0.05$ vs vehicle-treated J129.

Because vascular effects of the 2 doses tested were superimposable, results were cumulated. As shown in Figure 3A, capillary density of ischemic adductors was enhanced in R916-treated CD1 (1207 ± 46 versus 917 ± 112 cap/mm² in vehicle group, $P < 0.005$). Results were comparable when expressed as a capillary-to-myofiber ratio (Figure 3B). In contrast, capillarity of contralateral normoperfused non-injected muscles remained unaffected. Similar results were obtained in R916-treated J129, whereas, as expected, the agonist did not improve the deficient capillary response typical of B_1 knockouts (data not shown).

Effect of B_1 Manipulation on Post-Ischemic Perfusion Recovery

As depicted in Figure 4, CD1 showed an impaired hemodynamic recovery under chronic B_1 blockade by either R715 or DALBK. In fact, although 3 weeks were enough for vehicle-treated mice to recover, the limbs of antagonist-treated mice were still hypoperfused at the end of this time frame ($P < 0.01$).

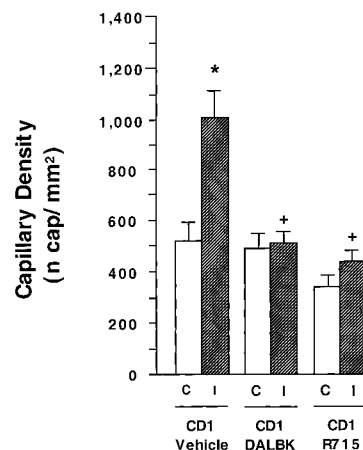


Figure 2. Bar graph shows the effect of B_1 receptor blockade on ischemia-induced vascularization in a different wild-type mouse strain (CD1). At day 21 after surgery, capillary response to ischemia was blunted by B_1 antagonism with DALBK or R715. Values are mean \pm SEM. Captions are the same as in Figure 1.

As shown in Figure 5, recovery of mice with B_1 genetic disruption was dramatically compromised, due to the virtually absent neovascularization (see above). Limb necrosis developed in the majority of B_1 knockouts, leading to spontaneous auto-amputation in 13 out of 15 animals (87 versus 29% in J129, $P < 0.01$). The 2 remainders showed persistent limb ischemia at 2 weeks from surgery, with a blood perfusion to the ischemic limb 69 and 63% less than that of contralateral side. In consideration of the severe outcome, animals were sacrificed at this stage to avoid unnecessary pain.

Conversely, local injection of the B_1 agonist R916 into the ischemic muscles of J129 improved clinical outcome ($P < 0.001$). Limb salvage occurred within 2 weeks in 12 (60%) out of 20 mice. Among remainders, 3 (15%) showed residual ischemia and 5 (25%) experienced foot necrosis. As shown in Figure 6, sequential analysis of limb blood flow denoted an accelerated recovery in R916-treated groups. With the highest dose, a beneficial effect was already detected at 1 week.

Effect of B_1 Signaling on EC Proliferation and Survival

Pharmacological manipulation of the B_1 affects HUVEC proliferation and apoptosis. After SI preconditioning, HUVEC spontaneous mitotic activity was higher (data not shown, $P < 0.05$) and the response to R916 increased (2.1-fold versus 1.5-fold in not SI in response to 100 nmol/L R916, $P < 0.05$), suggesting that the B_1 receptor might be up-regulated by SI. Figure 7 shows the dose-response to R916 after SI. The proliferative effect of R916 was prevented by R715 (Figure 7), whereas R715 per se was ineffective (data not shown).

In experiments performed at low serum concentration, R916 reduced apoptosis (Figure 8A and 8B), whereas R715 had no effect (data not shown). On the other hand, R715 increased apoptosis of HUVECs cultured under standard FBS concentration (Figure 8C and 8D), suggesting that B_1 ligands

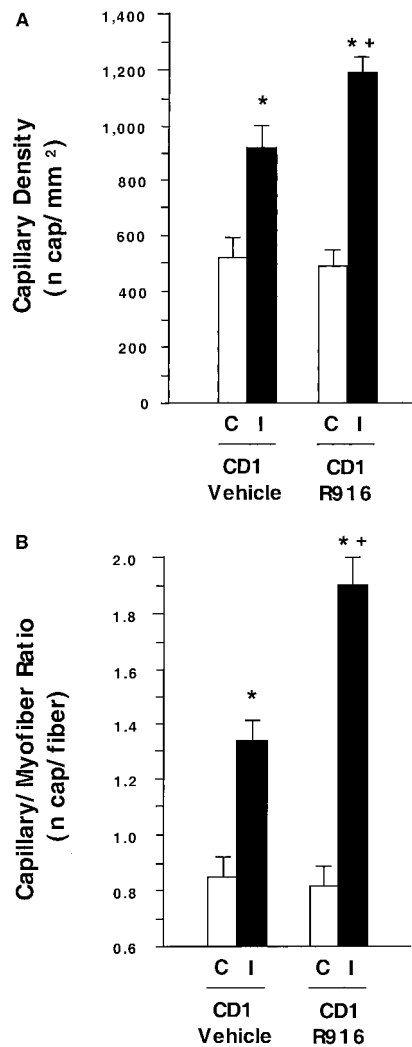


Figure 3. Bar graph shows the effect of B₁ agonism on reparative neovascularization. Local injection of the B₁ agonist R916 enhanced the native angiogenic response to ischemia as denoted by increased muscular capillary density (panel A) and capillary/myofiber ratio (panel B) in ischemic muscles (I, full columns), without altering capillarity of contralateral adductors (C, open columns). Values are mean \pm SEM. * $P < 0.05$ vs contralateral, ** $P < 0.05$ vs vehicle.

generated by ECs from serum-derived substrates may act autocrinally to favor cell survival.²² LDH levels did not differ among groups (data not shown), excluding a cytotoxic effect of R715. No differences were seen when cells were incubated under hypoxic conditions (data not shown).

Discussion

Circumstantial evidence derived from pharmacological studies suggests a role for the B₁ receptor in capillary proliferation.^{8,16–19} By exploiting the powerful tool of gene knockout strategy, we demonstrate that the endogenous B₁ signaling is indeed essential for developing new blood vessels with functional relevance for limb post-ischemic recovery. Another important finding of the present study is the discovery that B₁ stimulation by local administration of a specific receptor agonist has a potential in therapeutic angiogenesis.

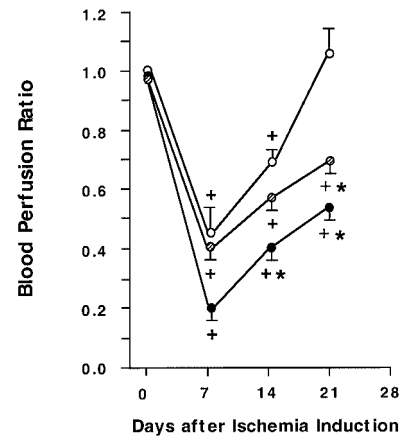


Figure 4. Line graph shows the effect of B₁ blockade on post-ischemic recovery. Vehicle-treated CD1 (open circles) showed restored perfusion after 21 days from surgery, whereas recovery was incomplete in mice given DALBK or R715 (full and hatched circles, respectively). Values are mean \pm SEM. * $P < 0.05$ vs time 0, ** $P < 0.05$ vs vehicle.

Increased kinin levels in the coronary sinus blood of dogs and humans have been reported after myocardial ischemia.^{23,24} For a long time, the compensatory relevance of this phenomenon has been attributed to the ability of kinins to cause vasodilation and preserve muscular energy content.^{25,26,27} More recently, the theory has been proposed that kinins may exert long-term protection on ischemic tissue by potentiation of neovascularization. We and others have documented that B₁ gene expression is activated in ischemic skeletal muscles and myocardium.^{8,15} Furthermore, in vitro studies showed that engagement of kinin B₁ by des-Arg¹⁰-kallidin or R916 stimulates proliferation of coronary ECs.^{16,17} We found that the mild proliferative effect of R916 on HUVECs under normal conditions is potentiated by preconditioning with a culture system mimicking an ischemic milieu. This finding favors possible implication of the B₁ in the EC program leading to capillarization in response to an ischemic injury. Furthermore, we demonstrated that the B₁ plays a role in EC survival.

Seminal studies from our group showed that B₁ pharmacological blockade inhibits capillary proliferation in response to limb ischemia.⁸ This assumption was confirmed by the use of 2 different antagonists and replicated in both wild-type J129 and CD1 strains. Moreover, we provided the new information that B₁ antagonism severely impairs blood perfusion recovery of ischemic muscles. Another major novelty of the present study is the observation that genetic disruption of the B₁ results in the absolute failure to mount reparative angiogenesis. The vascular deficit led to an unusual incidence of limb necrosis and auto-amputation. Congenital absence of the receptor could account for the severe outcome of B₁ knockouts compared with that of antagonist-treated animals. Moreover, the mild reduction in capillarity that was evidenced in not ischemic limbs of knockouts calls for further studies aimed to assess a potential role of B₁ in vasculogenesis.

Besides acting as direct modulators of EC growth and survival, kinins may participate in post-ischemic neovascu-

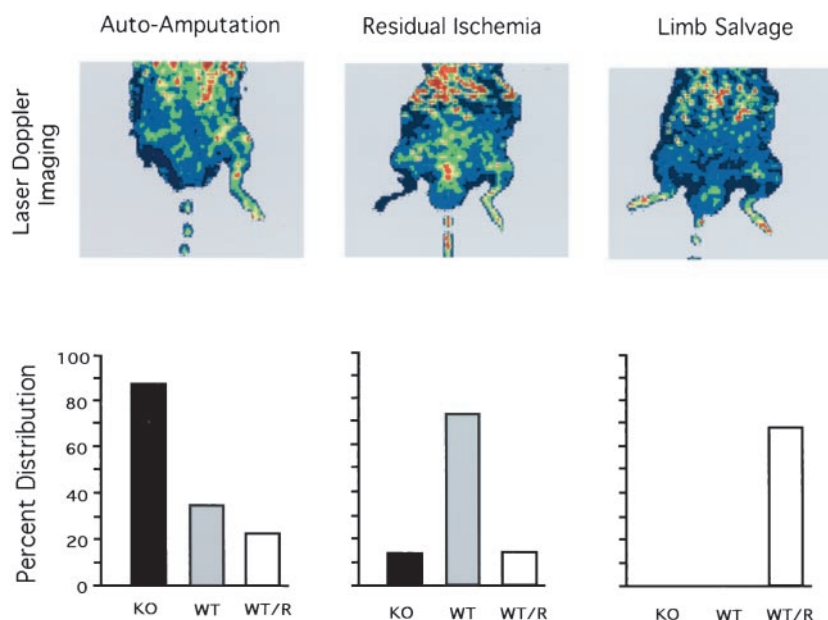


Figure 5. Upper panels show representative laser Doppler images of 3 different outcomes observed at 14 days from induction of ischemia. Left, auto-amputation of the ischemic limb occurring with higher incidence in B_1 knockouts. Middle, persistent ischemia without leg loss occurring especially in untreated wild-type J129 (WT). Right, limb salvage with complete restoration of perfusion observed only in R916-treated WT (WT/R). Lowest blood flow is indicated in black or dark blue, highest flow in yellow or red, and intermediate grading in green. Lower panels report percent distribution of above outcomes. Differences in distribution among groups were evaluated by χ^2 analysis.

larization by increasing extravasation of plasma proteins that would provide a provisional scaffold for growing vasculature. Furthermore, kinins are potent attractants for leukocytes, which represent an additional source of EC growth factors, as well as kallikrein and kininogen, ie, the components required for kinin generation.^{6,28} Interestingly enough, B_1 knockouts feature a drastic reduction in the extravasation of leukocytes in inflamed tissues,²⁰ a deficit that may eventually contribute in impaired neovascularization.

Another intriguing possibility regards coupling of B_1 receptor to neuronal modulation of angiogenesis, as supported by the observation that kinin challenge stimulates non-adrenergic non-cholinergic C-fibers to release the pro-angiogenic tachykinin neuropeptides.²⁹ Accordingly, the alteration in C-fiber trafficking that has been reported in B_1 knockouts²⁰ may contribute in impaired neovascularization.

The present study clearly indicates that ligands activating the B_1 possess a significant therapeutic potential for the

treatment of peripheral ischemia. Interestingly, R916 also increases capillarity of normoperfused muscle (Costanza Emanuelli, unpublished results, 2001), suggesting a potential application in patients experiencing intermittent ischemia. Administration of B_1 agonists could be safer than adenovirus-mediated growth factor gene delivery that reportedly produces harmful side effects mainly attributed to viral vectors. Furthermore, receptor agonists are devoid of immunogenic activity. This fact represents another important advantage over adenovirus-mediated gene therapy, whose immunogenicity precludes long-term duration of transgene expression as well as effectiveness in case of readministration.

It could be argued that uncontrolled B_1 activation might trigger local or systemic inflammatory reactions in susceptible individuals. However, such a strategy is made less risky by the availability of potent receptor antagonists. Further-

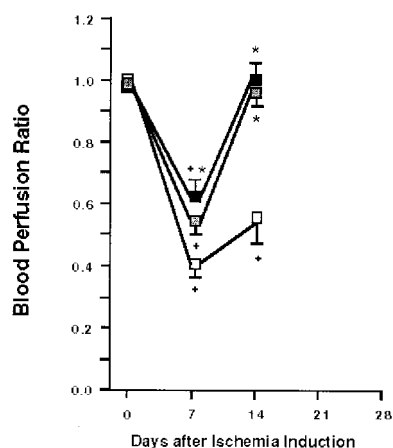


Figure 6. Line graph shows the effect of R916 agonist on post-ischemic recovery. Perfusion recovered more rapidly in CD1 given R916 once or twice daily (hatched and full squares, respectively) compared with vehicle (open squares). Values are mean \pm SEM. * P < 0.05 vs time 0, * P < 0.05 vs vehicle.

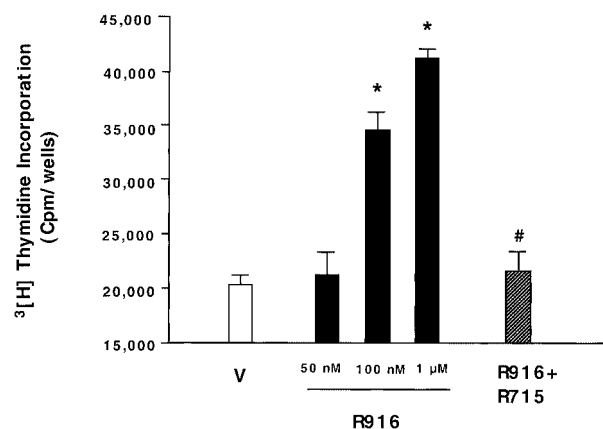


Figure 7. Bar graph shows the effect of vehicle (V, open column) or increasing concentrations of R916 (full columns) on ^3H thymidine incorporation by HUVECs, preconditioned with simulated ischemia (SI). R916 enhanced the proliferative response to SI. The effect of R916 (100 nmol/L) was prevented by R715 antagonist (1 μ mol/L, hatched column). Values are mean \pm SEM. * P < 0.05 vs vehicle; # P < 0.05 vs R916 (100 nmol/L).

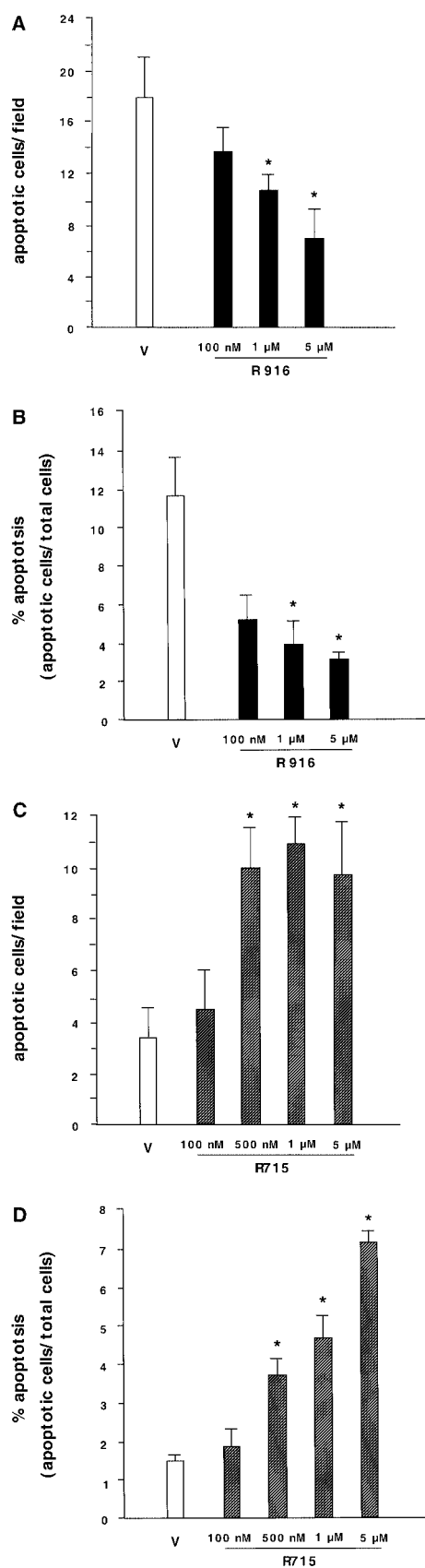


Figure 8. Bar graphs show HUVEC apoptosis under low (A and B) or normal (C and D) serum conditions. When cells were incubated with low serum, experiments were terminated after 24h, otherwise after 48h. Apoptosis is expressed as the number

more, because of their ability to interfere with the angiogenic program, B₁ receptor antagonists might be envisaged not only as kinin antidotes but also as therapeutic reagents to combat the pathological angiogenesis in cancer and chronic inflammatory diseases. Thus, B₁ pharmacological manipulation might open new avenues for the treatment of a wide range of diseases characterized by impaired or excessive vascular growth.

Acknowledgments

This work was supported by a grant from the Juvenile Diabetes Research Foundation (JDRF, no. 2000/49), USA to Dr Madeddu and by a PROBRAL grant of the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the German Academic Exchange Service to Dr Bader and Dr Pesquero. During a limited period of the study, Dr Emanuelli was a visiting scientist at the Cardiovascular Research Laboratory of the St Elizabeth's Medical Center of Boston, Mass, sponsored by the Italian Society of Cardiology. Dr Cayla is a recipient of a MDC fellowship. The support of Dr Dirk Walter for counting apoptosis is acknowledged.

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of apoptotic cells per field (panels A and C) or as percent (panels B and D). HUVECs were incubated with vehicle (V, open columns), R916 (100 nmol/L to 5 μ mol/L, full columns), or R715 (100 nmol/L to 5 μ mol/L, hatched columns). Values are mean \pm SEM. * P < 0.05 vs vehicle.

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