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B1 receptor involvement in the effect of bradykinin on venular endothelial cell proliferation and potentiation of FGF-2 effects

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1 Bradykinin (BK) contributes to the inflammatory response inducing vasodilation of postcapillary venules and has been demonstrated to induce neovascular growth in subcutaneous rat sponges.

2 In this study the ability of BK to stimulate cell growth and migration in cultured endothelium from coronary postcapillary venules (CVEC) has been investigated.

3 [^{3}H]-thymidine incorporation in subconfluent and synchronised CVEC was used to monitor DNA synthesis over 24 h. BK promoted a concentration-dependent increase of DNA synthesis with maximal activity at 100 nM. At this concentration BK also induced 18 fold accumulation of c-Fos protein immunoreactivity in the nucleus within 1 h from peptide exposure.

4 The total number of cells recovered after 48 h exposure to BK was increased in a concentration-dependent manner. Maximal effect was produced by 100 nM concentration of the peptide which produced 50% increase in cell number. The selective B1 receptor agonist Des-Arg9-BK mimicked the proliferative effect of BK, while the B2 receptor agonist kallidin was devoid of any activity. The proliferation induced by BK was abolished in a concentration-dependent manner by the addition of the B1 selective antagonist Des-Arg9-Leu8-BK, while the selective B2 receptor antagonist HOE140 did not modify BK-induced growth.

5 DNA synthesis and growth promoted by a threshold concentration of fibroblast growth factor-2 (FGF-2) (0.25 nM) were potentiated by increasing concentrations of BK and Des-Arg9-BK.

6 Endothelial cell migration assessed by the Boyden Chamber procedure was not promoted by BK or the selective B1 and B2 receptor agonists.

7 These data are the first demonstration that BK promotes growth of endothelial cells from postcapillary venules. The mitogenic activity of BK involves c-Fos expression and potentiates the growth promoting effect of FGF-2. Only the B1 receptor appears to be responsible for the proliferation induced by BK and suggests that this type of receptor might be implicated in favouring angiogenesis of coronary venules.

Keywords: bradykinin; B1 and B2 receptors; postcapillary endothelial cells; proliferation; migration; fibroblast growth factor-2.

Introduction

Kinins are potent vasodilating peptides that are released from precursor kininogens by kallikreins. Kinins bind to receptors and mediate a broad spectrum of biological effects including vasodilation, smooth muscle contraction and relaxation, pain and inflammation. Bradykinin (BK) is a potent inflammatory mediator capable of inducing vasodilation and dramatically enhancing the transport of water and proteins across postcapillary venules by its action on endothelial cells.

Increasing evidence demonstrates that vasoactive peptides which control vascular tone also contribute to changes in cell growth. In particular BK has been shown to possess opposite effects on cell proliferation. In smooth muscle cells BK blocks spontaneous and growth factor-induced cell proliferation (Patel & Schrey, 1992; Yau *et al*., 1996; Dikson & Dennis, 1997), while in other cell types a growth promoting effect of BK has been described (Goldstein & Wall, 1984; Marceau & Tremblay, 1986; Kimball & Fisher, 1988; el-Dahr *et al*., 1996).

Angiogenesis is the process of new vessel generation which leads to neovascularization (Folkman, 1982). In the adult tissue, angiogenesis is of importance for various physiological and pathological processes such as ovulation and corpus luteum formation, healing processes including recovery from myocardial infarction, tumour growth and metastasis, inflammation, chronic arthropathies and diabetic retinopathies. The morphogenesis of capillaries involves a concerted sequence of events usually described as migration of the endothelium in a controlled direction, cellular proliferation and maturation (Ziche *et al*., 1996). The promotion or inhibition of angiogenesis has potentially important therapeutic applications.

Fan and coworkers (Hu & Fan, 1993; Hu *et al*., 1995) reported on angiogenesis induced *in vivo* by BK in synergism with interleukin-1 (IL-1). The receptor subtype involved in BK-induced neovascular response and the mechanism by which BK induced angiogenesis however were not elucidated. The aim of the present study was to assess whether BK could directly affect endothelial cell growth and migration, which are necessary steps for angiogenesis. Endothelial cells from postcapillary venules were selected for this study since these vessels are the primary site of BK effects as well as of neovascular growth (Folkman & Shing, 1992). Furthermore, we attempted to characterize the BK receptor subtypes present in cultured coronary venular endothelial cells and their role in mediating BK effects.

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Methods

Cell line and culture conditions

The coronary venular endothelial cells (CVEC) were obtained by a bead-perfusion technique through the bovine coronary sinus as previously reported (Schelling *et al*., 1988). The endothelial nature of the cells was evidenced by labelling with antibody to Factor VIII-related antigen and uptake of acetylated low-density lipoproteins. At confluence, they form a typical contact-inhibited monolayer with the usual cobblestone morphology. Cells were maintained in culture in DMEM supplemented with 10% bovine calf serum (CS, Hyclone) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) on gelatin coated dishes. Cells were cloned and each clone was subcultured up to a maximum of 25 passages. Passages between 15 and 20 were used in these experiments.

c-Fos immunohistochemistry

2500 cells were seeded onto 96 multiwell plates in DMEM containing 10% serum and let adhere overnight. Cells were starved from serum for 3 days. Treatment with test substances was performed in 0.5% serum at 37°C for the indicated times. Cells were washed with phosphate buffered saline (PBS) and fixed in 2% formaldehyde in PBS at room temperature for 20 min. Fixative was removed by three washes with PBS containing 10 mM glycine. The cells were permeabilized for 5 min with 1% Nonidet P-40 in PBS-glycine and then incubated at 4°C overnight with polyclonal sheep c-Fos peptide antiserum (Genosys, 1 : 500 dilution in PBS containing 1% of normal horse serum). The bound antibody was detected with biotinylated anti-sheep IgG and the avidin-biotin peroxidase (ABC, Vector Laboratories) according to the manufacturer's instructions and visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride (0.3 mg/ml) and hydrogen peroxide (0.015%) for 4–8 min at room temperature. The number of labelled nuclei was counted in a blinded manner at 10 × magnification in three random fields/well with the aid of an ocular grid.

[^{3}H]-Thymidine incorporation

DNA synthesis was quantified by [^{3}H]-thymidine incorporation of subconfluent and synchronized cell monolayers (Ziche *et al*., 1994). CVEC were seeded onto 24 multiwell plates (1×10^4 cells/well) in DMEM supplemented with 5% CS and were let to adhere overnight. After 48 h in serum-free media (0.1% CS medium), cells were incubated with increasing concentrations of the agents for 24 h and pulsed for 1 h with 0.5 μCi [^{3}H]-thymidine per well. DNA was precipitated with 5% trichloroacetic acid and extracted with 0.3 M NaOH and the recovered radioactivity measured. Data are expressed as percent thymidine incorporation of basal response.

Proliferation studies

For the proliferation studies, the same protocol as for [^{3}H]-thymidine incorporation was followed (Ziche *et al*., 1994). In the experiments with selective B1 and B2 receptor antagonist, cells were pretreated with antagonists 15 min before the addition of BK. After 48 h incubation the supernatants were removed from the multiwell plates. Cells were fixed with methanol and stained with Diff-Quik (Dade International). Cell numbers were obtained by counting in seven random fields at a magnification of 100 with the aid of an ocular grid (21 mm^2). Data are expressed as percent of basal response.

Migration assay

The Boyden Chamber procedure was used to evaluate cell migration (Ziche *et al*., 1994). The method is based on the passage of endothelial cells across porous filters against a concentration gradient of the migration effector. The Neuro Probe 48-well micro-chemotaxis chamber (Nuclepore) was used. The two wells were separated by a polyvinyl-pyrrolidone-free polycarbonate filter, 8 μm pore size, coated with type I collagen (100 μg/ml) and fibronectin (10 μg/ml). Test solutions were dissolved in 0.1% CS medium and placed in the lower wells. Fifty μl of cell suspension (1.2×10^4 cells) were added to each upper well. The chamber was incubated at 37°C for 4 h and the filter was then removed and fixed in methanol. Cells migrated on the lower surface of the filter were stained with Diff-Quik (Dade International) and counted using a light microscope at a magnification of 400 in 10 random fields per each well. Each experimental point was done in triplicate. Migration was expressed as the number of total cells counted per experiment.

Urokinase-type plasminogen activator (uPA) assay

The assay was performed on subconfluent cultured cells as described (Ziche *et al*., 1997). Briefly, CVEC were plated in 24-well dishes at 5×10^4 cells/cm^2. After 24 h, cells were washed twice with serum-free medium and incubated in fresh medium containing 0.4% CS and different concentrations of test substances. 24 h later, cell-associated uPA activity was measured. To this purpose, cells were washed twice with PBS and extracted with 100 μl of 0.05% Triton X-100 in 60 mM Tris-HCl, pH 8.5 (T/T buffer). Aliquots of the cell extracts corresponding to 1 μg of protein were incubated in a microtiter plate with 4.2 ng of purified glu-plasminogen (Kabi AB) and 42 nmol of plasmin chromogenic substrate H-D-norleucyl-hexahydrotyrosyl-lysine-p-nitroanilide-acetate (American Diagnostics) in 150 μl of T/T buffer. After incubation at 37°C, the plate was read at 405 nm with an automatic microplate reader. Human urokinase (Calbiochem) was used as a standard.

Cyclic AMP measurements

Cyclic AMP (cAMP) levels were measured as previously reported (Ziche *et al*., 1993). Cell monolayers were pretreated for 30 min with 10 μM indomethacin and 50 μM 3-isobutyl-5-methyl-xanthine and stimulated with test substances for 5 min. At the end of incubation cells were scraped off in 0.5 ml ice-TCA (10%, w/v). After centrifugation at 1500 r.p.m. for 5 min, pellets were resuspended with 0.5 M tri-n-octylamine dissolved in 1,1,2 trichloro-trifluoroethane (Sigma). The levels of cAMP in the aqueous phase were measured by commercially available radioimmunoassays in duplicate with iodinated tracers from Amersham. Results are expressed as pmol/mg protein.

Reagents

Bradykinin, Des-Arg9-BK, Des-Arg9-Leu8-BK, Kallidin were from Sigma. HOE140 was kindly provided by Menarini Pharmaceuticals, Florence, Italy. Fibroblast growth factor-2 (FGF-2) was from Pre-Protech, Inalco, Milan, Italy.

Statistical analysis

Results are expressed as means ± s.e.m. for (n) experiments. Multiple comparisons were performed by one-way ANOVA and individual differences tested by Fisher's test after the demonstration of significant inter-group differences by ANOVA.

Results

Effect of BK on DNA synthesis, c-Fos activation and proliferation of microvascular endothelial cells

Subconfluent and synchronised endothelial cells were stimulated with increasing concentrations of BK (0.01 nM – 1 μM) in 0.1% CS and DNA synthesis was evaluated after 24 h exposure. BK induced a concentration-dependent thymidine incorporation, with maximal effect at 100 nM (50% increase over basal response). The EC_{50} was 0.2 ± 0.1 nM (Figure 1). The effect produced by the maximal effective concentration of BK overlapped with the amount of thymidine incorporation induced by FGF-2 (1 nM).

BK was evaluated for its ability to activate early gene expression. BK (100 nM) induced c-Fos activation producing a significant accumulation of protein immunoreactivity in the nucleus within 15 min (3.4 fold of basal control condition, Table 1). Maximal activation was observed at 1 h, producing an 18 fold increase of positive nuclei number, and after 3 h stimulation the immunoreactivity was still significantly different from basal levels (4 fold). FGF-2 (10 nM) activated c-Fos to a much smaller extent than BK, producing its maximal effect at 1 h (2 fold increase).

CVEC proliferation was also evaluated as the total number of cells recovered after 48 h exposure to BK. BK (1 – 100 nM) sustained cell growth in a concentration-dependent manner with maximal effect at 100 nM (148 ± 3 counted cells/well vs a basal value of 100 ± 4.3, $P < 0.05$) (Figure 2). These findings clearly indicate a positive correlation between the induction of DNA synthesis and cell proliferation.

Receptor subtype characterization

The receptor subtype involved in cell growth induced by BK was assessed by the use of the selective B1 and B2 receptor agonists, Des-Arg9-BK and kallidin, respectively. Cells were treated with increasing concentrations of the two peptides (1 – 100 nM) and cell growth was measured as total number of cells counted after 48 h incubation. The selective B1 receptor agonist Des-Arg9-BK reproduced the proliferative effect induced by BK, while the selective B2 receptor agonist kallidin did not (Figure 2).

To further characterize the receptor subtype activated by BK on CVEC, cells were treated with selective B1 and B2 receptor antagonists and then exposed to BK. The effect of BK on CVEC was reduced in a concentration-dependent manner by the pretreatment with the B1 receptor antagonist Des-Arg9-Leu8-BK. The maximal effective concentrations of the antagonist able to completely block BK-induced growth were 0.7 and 1 μM (Figure 3a). The antagonist alone did not

Table 1 Effect of BK in stimulating c-*fos* gene expression

	Time (min)			
	15	*60*	*120*	*180*
Basal	4 ± 1.4	26 ± 0.5	29.5 ± 5	40 ± 5.6
BK 100 nM	13.5 ± 9*	469 ± 48*	327 ± 50*	157 ± 31*
FGF-2 10 nM	7 ± 2.8	55 ± 15*	19.5 ± 6	62 ± 17

c-Fos protein accumulation in the nucleus was detected by immunohistochemistry in postcapillary endothelial cells exposed to 100 nM BK and 10 nM FGF-2 for different times. Data are expressed as total positive nuclei counted/well ($n = 4$ in triplicate). *$P < 0.01$ vs basal.

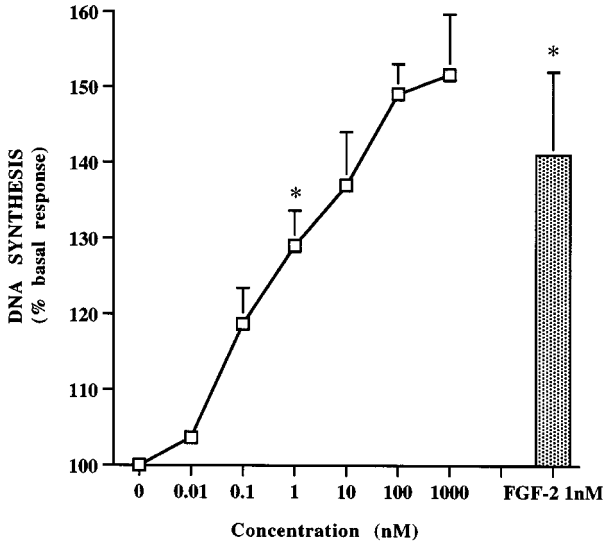

Figure 1 Effect of BK on DNA synthesis in postcapillary endothelial cells. [^{3}H]-thymidine incorporation was monitored following exposure of synchronized and serum-starved CVEC to increasing concentrations of BK for 24 h. DNA was precipitated, extracted and the recovered radioactivity measured. Data are expressed as % of basal response and represent the results of at least six experiments run in duplicate. Basal thymidine incorporation was 34345 ± 5250 recovered c.p.m./well. The first significant value is reported. *$P < 0.01$ vs basal.

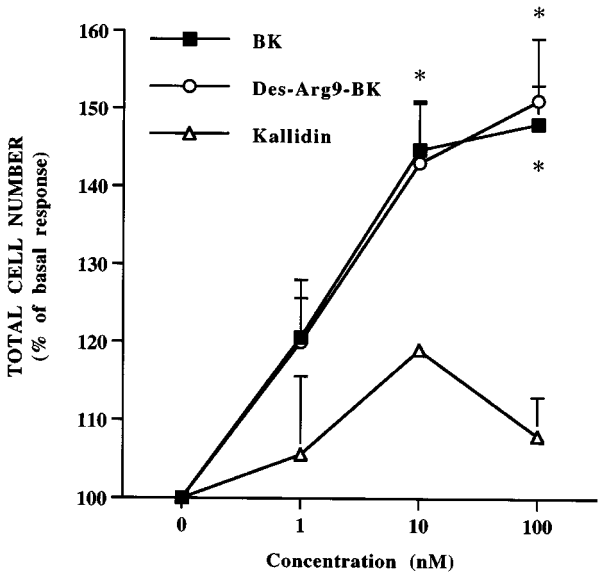

Figure 2 Effect of BK, selective B1 and B2 receptor agonists on postcapillary endothelial cell growth. The total number of cells recovered after 48 h exposure to BK, the selective B1 (Des-Arg9-BK) and B2 receptor (kallidin) agonists was counted. Proliferation is expressed as % of the basal response and represent the results of at least four experiments run in duplicate. Control basal value was 100 ± 4.3 counted cells/well. *$P < 0.01$ vs basal.

substantially modify basal proliferation(116 ± 5 and 107.8 ± 5 counted cells/well with 0.7 and 1 μM antagonist, respectively, vs a basal value of 110 ± 6). Trypan blue exclusion experiments did not reveal any cytotoxic effect by the antagonist.

In parallel experiments, the selective B2 receptor antagonist HOE140 (Hock *et al*., 1991) used at the concentration of 0.1 and 1 μM, did not significantly affect BK-induced proliferation (Figure 3b). The specificity of B1 antagonist was assessed on the growth of CVEC induced by a different endothelial cell mitogen, FGF-2. Des-Arg9-Leu8-BK at the dose of 1 μM did not modify FGF-2-induced proliferation (Figure 3a). The presence of B1 and B2 receptors in CVEC was confirmed by assessing the activation of adenylate cyclase following BK treatment in the presence of the selective B1 and B2 receptor antagonists. cAMP levels measured by radioimmunoassay increased by $53\pm15\%$ over basal when CVEC were exposed to 100 nM BK for 5 min (6.87 ± 1.7 pmol/mg protein in resting conditions; $n=3$ experiments). The preincubation for 15 min with 1 μM Des-Arg9-Leu8-BK abolished the accumulation of cAMP ($87.3\pm12.5\%$ inhibition, $P<0.01$), while 1 μM HOE 140 reduced BK-induced cAMP formation by $67.3\pm15.6\%$ ($P<0.05$). The antagonists did not affect basal cAMP levels.

Effect of BK on migration and uPA activity

During angiogenesis endothelial cells migrate and acquire an invasive capacity by increasing proteolytic activity. The effect of BK was then assayed on migration and uPA assays. BK ($0.1-100$ nM) had no effect on CVEC chemotaxis and uPA activity, while endothelial cells were highly responsive to FGF-2 in both assays (Figure 4a and b). Moreover, neither the B1 nor the B2 receptor agonist stimulated CVEC mobilization (Figure 4a).

BK and B1 receptor agonist potentiate FGF-2-induced proliferation

Different growth factors cooperate to promote neovascular growth. Thus, the effect of BK was assessed for its ability to synergize in promoting endothelial cell growth with the angiogenic factor FGF-2. FGF-2 produced concentration-dependent proliferation of CVEC with maximal effect at 1 nM (data not shown). The threshold concentration of 0.25 nM of FGF-2, which increased DNA synthesis and cell proliferation by approximately 125 and 20%, respectively, was used in these experiments.

When BK was administered to the cells together with FGF-2, the peptide did not further promote FGF-2-induced DNA synthesis (Figure 5a). Since data from early gene expression had indicated that BK rapidly activated c-Fos in CVEC, we postulated that transient exposure of the cells to the peptide could trigger the [^{3}H]-thymidine incorporation and reproduce

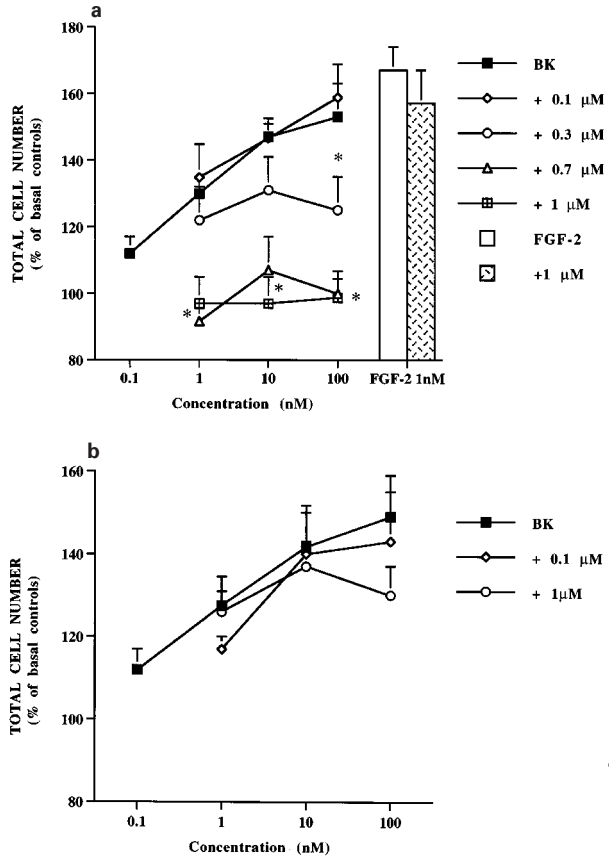

Figure 3 Effect of selective B1 (a) and B2 (b) receptor antagonists on BK-induced proliferation. The total number of cells was counted after 48 h exposure to BK in cells treated with different concentrations of the selective B1 (Des-Arg9-Leu8-BK) (panel a) and B2 (HOE140) (panel b) receptor antagonist. Data are expressed as % of the effect of antagonists alone, and represent the results of at least four experiments run in duplicate. Control basal value was 110 ± 6 counted cells/well. $^*P<0.01$ vs BK alone.

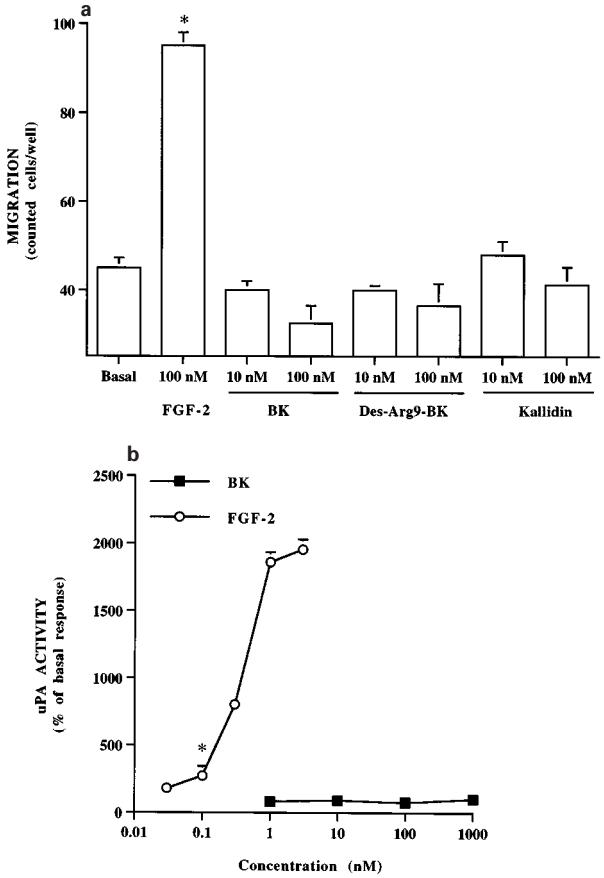

Figure 4 Effect of BK, selective B1 and B2 receptor agonists and FGF-2 on the migration (a) and uPA activity (b) of postcapillary endothelial cells. (a) The 48-well microchemotaxis chamber was used to assess migration. The chamber was incubated at 37°C for 4 h. Data are means$\pm$s.e.m. from at least four experiments run in triplicate. (b) Cell-associated uPA activity was measured in subconfluent CVEC after 24 h exposure to increasing concentrations of FGF-2 or BK, by a spectrophotometric assay using a plasmin chromogenic substrate. Data are means$\pm$s.e.m. from at least two experiments run in triplicate. The first significant value is reported. $^*P<0.01$ vs basal.

a better setting to test the potentiating effect of FGF-2. Increasing concentrations of BK added to the supernatant for 30 min and removed, induced an increase of DNA synthesis although the extent of the response was 3 fold lower compared to that obtained in the continuous presence of the peptide (Figure 5). In cells which received increasing concentrations of BK for 30 min and fresh medium containing 0.25 nM FGF-2, a potentiation of DNA synthesis with a synergistic effect was observed. BK effect was evident at all concentrations tested. After the transient exposure to 0.01 and 0.1 nM BK, DNA synthesis by FGF-2 was potentiated by 1.25 and 1.75 fold, respectively (Figure 5b).

Similar results were obtained evaluating cell growth as total cell number after 48 h incubation. Submaximal concentration of both BK and the selective B1 agonist (1 nM) was able to cooperate with FGF-2 producing an additive effect (Table 2). Transient exposure of the cells to BK or to the B1 agonist for 30 min was sufficient to potentiate FGF-2 effect. Simultaneous addition of the growth factor and the peptides to the cells revealed that the B1 agonist was effective in potentiating FGF-2 effect while the natural peptide was not (Table 2).

Table 2 Potentiation of BK and selective B1 receptor agonist (Des-Arg9-BK) with FGF-2 in proliferation

	Tested peptide	
Treatment	*BK*	*Des-Arg9-BK*
None	100	100
Peptide 30′→FGF-2	151.6±6#	149±7#
Peptide+FGF-2	124±5.5*	155±9#
Peptide	122±2.7*	131.3±5*
FGF-2	125±5*	123±5#

The potentiation in CVEC proliferation by BK and FGF-2 was assessed by two experimental approaches: (a) BK or the B1 agonist (1 nM each) was given for 30 min, then removed and substituted with a threshold concentration of FGF-2 (0.25 nM), (b) BK or the B1 agonist was given simultaneously with FGF-2. Cellular proliferation was evaluated by the number of total cells counted after 48 h incubation. Data are expressed as % increase over basal response and represent the results of at least three experiments run in triplicate. Control basal value was 117±3.5 counted cells/well. *P<0.05 vs basal and #P<0.05 vs FGF-2 alone.

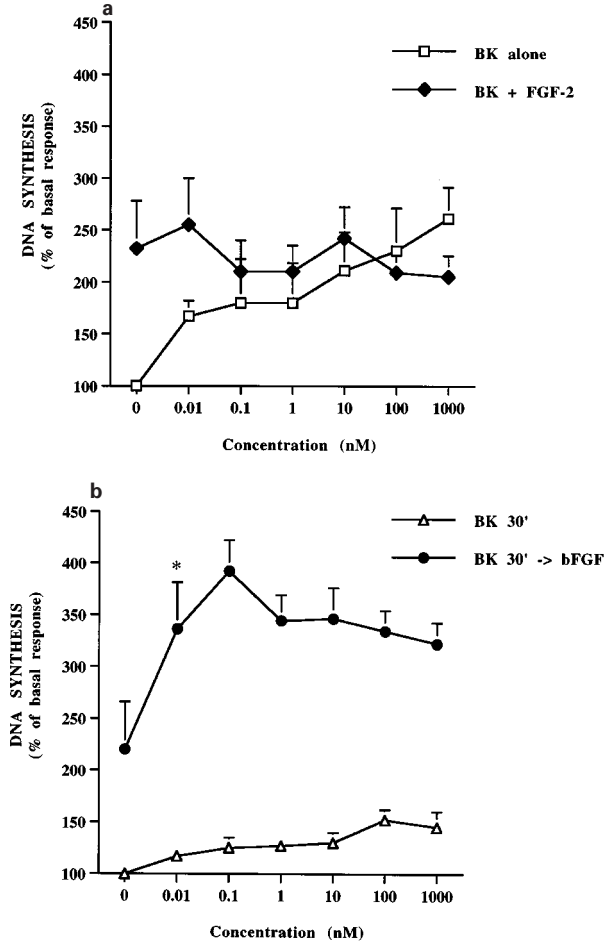

Figure 5 Potentiating effect of BK and FGF-2 on DNA synthesis of postcapillary endothelial cells. DNA synthesis was quantified by [^{3}H]-thymidine incorporation of subconfluent and synchronized cell monolayers. Experimental protocol: in panel (a) BK alone: cells were treated with BK for 24 h; BK+FGF-2: increasing concentrations of BK and 0.25 nM FGF-2 were given simultaneously. In panel (b) BK 30′→FGF-2: BK was given for 30 min, removed by aspiration and fresh medium containing 0.25 nM FGF-2 was added; BK 30′: cells were treated with BK for 30 min, the supernatant was removed and substituted with medium alone. Data are expressed as per cent thymidine incorporation of basal response. Data are the results of six experiments (run in duplicate) different from the ones reported in Figure 1. The first significant value is reported. *$P<0.01$ vs FGF-2 alone.

Discussion

This study was aimed to investigate by which mechanism BK contributed to angiogenesis. We demonstrate for the first time that BK exerts a specific and direct growth promoting effect on coronary postcapillary endothelial cells while it does not induce their migration. Early gene expression of c-*fos* is substantially increased by BK exposure in parallel with DNA synthesis. Nanomolar concentrations of BK favour the growth of cultured endothelium whose extent is similar to that produced by the angiogenic protein FGF-2. We document that this effect is concentration-dependent and is mediated by B1 receptor activation and that threshold concentrations of BK, through B1 receptor activation, synergistically increase FGF-2-induced endothelial cell proliferation.

The role of bradykinin in angiogenesis was demonstrated by Hu and coworkers by showing that *in vivo* neovascularization induced by IL-1 was potentiated by BK and abolished by a selective antagonist for the B1 receptor (Hu & Fan, 1993; Hu *et al*., 1995). Angiogenesis occurs as the end result of a cascade of interdependent steps leading to endothelial cell migration, growth and organization into a new capillary bud. Although the growth promoting effect of BK on different cell types has been reported in the literature, no information was available on the effect of BK on endothelial cells. Our data are the first demonstration that BK promotes the proliferation of endothelial cells. These data indicate that the contribution of BK to angiogenesis is direct and linked to the specific effect of the peptide on the growth of postcapillary endothelium since BK does not affect endothelial migration and uPA activity.

The receptor involved in BK-induced endothelial cell proliferation was characterized. Two subtypes of bradykinin receptors, B1 and B2 have been defined based on their pharmacological properties (Regoli & Barabe, 1980; Hall, 1992; Regoli *et al*., 1994). In physiological conditions the B2 receptor is constitutively expressed at vascular level. The B1 receptor expression, undetectable in physiological conditions, is found strongly up-regulated following tissue injury and inflammation (hyperemia, exudation, hyperalgesia etc.) (Schnerck *et al*., 1994; Drummond & Cocks, 1995). Our findings demonstrate that in postcapillary venular endothelial

cells both B1 and B2 receptors are present and that activation of only the B1 receptor is required to promote endothelial cell growth. The B1 receptor agonist Des-Arg9-BK is able to mimic BK-induced proliferation, while the B2 receptor agonist kallidin is devoid of any activity. Consistent with these data the selective B1 receptor antagonist Des-Arg9-Leu8-BK blocks endothelial cell proliferation promoted by BK while it does not affect FGF-2 action on endothelial cell growth. Conversely, the highly specific B2 receptor antagonist, HOE140 (Hock *et al*., 1991), does not modify BK-induced responses.

Multiple factors contribute to angiogenesis *in vivo*. Hu and coworkers described that *in vivo* IL-1 synergized with BK in promoting angiogenesis. This effect was apparently linked to B1 receptor induction by the IL-1 treatment since it was sensitive to a B1 receptor antagonist. Here we demonstrate that FGF-2 and BK synergize in producing DNA synthesis on endothelial cell, inducing an additive effect on cell growth. BK as well as the B1 agonist Des-Arg9-BK are able to cooperate with FGF-2. The lack of effect of the B1 antagonist on FGF-2 action confirms that in coronary postcapillary endothelium BK and FGF-2 are independent promoter of cell growth. Preincubation with either BK or the selective B1 agonist is able to increase cell proliferation in response to FGF-2 in an additive manner, further indicating that the B1 and FGF-2 receptors are coupled to different signal transduction pathways. In keeping with these observations it is interesting to note that a substantial activation of c-Fos occurs very rapidly in response to BK, while FGF-2 appears a weak activator of the early gene. This rapid activation induces endothelial cells to increase DNA synthesis and to be more responsive to FGF-2. The different behaviour of BK and the B1 agonist in potentiating FGF-effect when added together could be due to fact that the B1 agonist is a full agonist and can provide a more stable activation of B1 receptor when compared to the natural peptide. Conversely, a reduction of the BK concentration needed to activate the B1 receptor can occur when BK and FGF-2 are given in combination, since FGF-2 increases protease activity in CVEC, an event which can potentially reduce BK half-life.

Angiogenesis plays a crucial role in different pathological and physiological processes including wound healing and the recovery from myocardial infarction. Proliferation of coronary endothelial cells in the border zone of the ischemic myocardium contributes to limit the damage to the myocardium and the infarct size by favouring angiogenesis (Granger *et al*., 1994). It has been reported that brief periods of myocardial ischemia are cardioprotective (Yellon *et al*., 1993). Increased levels of bradykinin are produced as a result of the ischemic damage together with other vasoactive agents (as adenosine, angiotensin, endothelin) which can affect the neovascular processes (Dusseau *et al*., 1986; Noda *et al*., 1993; Le Noble *et al*., 1993; Hu & Fan, 1993; Morbidelli *et al*., 1995; Cohen & Downey, 1996). Our results suggest that on the coronary endothelium of postcapillary venules kinins exert a trophic effect which may result in a protective action of ischemic heart disease (Vegh *et al*., 1991; Starkopf *et al*., 1997). The direct effect of BK on coronary endothelium shown here provides a further evidence in support of the cardioprotective effect of ACE inhibitors *in vivo* through their action on endogenous kinins (Martorana *et al*., 1990; Noda *et al*., 1993; Schriefer *et al*., 1997). In conclusion our findings indicate a role for BK on coronary postcapillary endothelial cell proliferation relevant for the promotion of angiogenesis. Pharmacological agents mimicking BK effect linked to B1 receptor activation such as a B1 agonist or PKC inducer, and favouring BK action such as ACE inhibitor, have the potential to confer cardioprotection by inducing angiogenesis in ischemic heart diseases or during revascularization surgery.

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References

COHEN, M.V. & DOWNEY, J.M. (1996). Myocardial preconditioning promises to be a novel approach to the treatment of ischemic heart disease. *Annu. Rev. Med.* **47,** 21 – 29.

DIXON, B.S. & DENNIS, M.J. (1997). Regulation of mitogenesis by kinins in arterial smooth muscle cells. *Am. J. Physiol.,* **273,** C7 – C20.

DRUMMOND, G.R. & COCKS, T.M. (1995). Endothelium-dependent relaxations mediated by inducible B1 and constitutive B2 kinin receptors in the bovine isolated coronary artery. *Br. J. Pharmacol.,* **116,** 2473 – 2481.

DUSSEAU, J.W., HUTCHINS, P.M. & MALBASA, D.S. (1986). Stimulation of angiogenesis by adenosine on chick chorioallantoic membrane. *Circ. Res.,* **59,** 163 – 170.

EL-DAHR, S.S., DIPP, S., YOSIPIV, I.V. & BARICOS, W.H. (1996). Bradykinin stimulates c-*fos* expression, AP-1-DNA binding activity and proliferation of rat glomerular mesangial cells. *Kidney Int.,* **50,** 1850 – 1855.

FOLKMAN, J. (1982). Angiogenesis: initiation and control. *Ann. N.Y. Acad. Sci.,* **401,** 212 – 227.

FOLKMAN, J. & SHING, Y. (1992). Angiogenesis. *J. Biol. Chem.,* **267,** 10931 – 10934.

GOLDSTEIN, R.H. & WALL, M. (1984). Activation of protein formation and cell division by bradykinin and Des-Arg9-bradykinin. *J. Biol. Chem.,* **259,** 9263 – 9268.

GRANGER, H.J., ZICHE, M., HAWKER, J.R. Jr, MEININGER, C.J., CZISNY, L.E. & ZAWIEJA, D.C. (1994). Molecular and cellular basis of myocardial angiogenesis. *Cell. Mol. Biol. Res.,* **40,** 81 – 85.

HALL, J.M. (1992). Bradykinin receptors: pharmacological and biological roles. *Pharmac. Ther.,* **56,** 131 – 190.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, ST. BREIPOHL, G., KONIG, W., KNOLLE, J. & SCHOLKENS, B.A. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: *in vitro* studies. *Br. J. Pharmacol.,* **102,** 796 – 773.

HU, D.-E., & FAN, T.-P.D. (1993). [Leu8]des-Arg9-bradykinin inhibits the angiogenic effect of bradykinin and interleukin-1 in rats *Br. J. Pharmacol.,* **109,** 14 – 17.

HU, D.-E., HILEY, C.R., SMITHER, R.L., GREESHAM, G.A. & FAN, T.-P.D. (1995). Methods in laboratory investigation. Colleration of ^{133}Xe clearance, blood flow and histology in rat sponge model of angiogenesis. *Lab. Invest.,* **72,** 601 – 610.

KIMBALL, E.S. & FISHER, M.C. (1988). Potentiation of IL-1-induced BALB/3T3 fibroblast proliferation by neuropeptides. *J. Immunol.,* **141,** 4203 – 4208.

LE NOBLE, F.A.C., SCHREURS, N.H.J.S., VAN STRAATEN, H.W.M., SLAAF, D.W. & STRUIJKER-BOUDIER, H.A.J. (1993). Evidence for a novel angiotensin II receptor involved in angiogenesis in chick embryo chorioallantoic membrane. *Am. J. Physiol.,* **264,** R460 – R465.

MARCEAU, F. & TREMBLAY, B. (1986). Mitogenic effect of bradykinin and Des-Arg9-bradykinin on cultured fibroblasts. *Life Sci.,* **39,** 2351 – 2358.

MARTORANA, P.A., KETTENBACH, B., BREIPOHL, G., LINZ, W. & SCHOLKENS, B.A. (1990). Reduction of infarct size by local angiotensin-converting enzyme inhibition is abolished by a bradykinin antagonist. *Eur. J. Pharmacol.,* **182,** 395–396.

MORBIDELLI, L., ORLANDO, C., MAGGI, C.A., LEDDA, F. & ZICHE, M. (1995). Proliferation and migration of endothelial cells is promoted by endothelins by activation of ET_B receptors. *Am. J. Physiol.,* **269,** H686–H695.

NODA, K., SASAGURI, M., IDEISHI, M., IKEDA, M. & ARAKAWA, K. (1993). Role of locally informed angiotensin II and bradykinin in the reduction of myocardial infarct size in dogs. *Cardiovasc. Res.,* **27,** 334–340.

PATEL, K.V. & SCHREY, M.P. (1992). Inhibition of DNA synthesis and growth in human breast stromal cells by bradykinin: evidence for independent roles of B1 and B2 receptors in the respective control of cell growth and phospholipid hydrolysis. *Cancer Res.,* **52,** 334–340.

REGOLI, D. & BARABE, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.,* **32,** 1–46.

REGOLI, D., GOBEIL, F., NGUYEN, Q.T., JUKIC, D., SEOANE, P.R., SALVINO, J.M. & SAWUTZ, D.J. (1994). Bradykinin receptor types and B2 subtypes. *Life Sci.,* **55,** 735–749.

SCHELLING, M.E., MEININGER, C.J., HAWKER, J.R. & GRANGER, H.J. (1988). Venular endothelial cells from bovine heart. *Am. J. Physiol.,* **254,** H1211–H1217.

SCHNERCK, K.A., HESS, J.F., STONESIFER, G.Y. & RANSOM, R.W. (1994). Bradykinin B1 receptors in the rabbit aorta smooth muscle cells in culture. *Eur. J. Pharmacol.,* **266,** 277–282.

SCHRIEFER, J.A., BROUDY, E.P. & HASSEN, A.H. (1997). Endopeptidase inhibitors decrease myocardial ischemia/reperfusion injury in an in vivo rabbit model. *J. Pharmacol. Exp. Ther.,* **278,** 1034–1039.

STARKOPF, J., BUGGE, E. & YTREHUS, K. (1997). Preischemic bradykinin and ischaemic preconditioning in functional recovery of the globally ischaemic rat heart. *Cardiovasc. Res.,* **33,** 63–70.

VEGH, A., SZEKERES, L. & PARRAT, J. (1991). Local intracoronary infusion of bradykinin profoundly reduces the severity of ischemia-induced arrhythmias in anaesthetized dogs. *Br. J. Pharmacol.,* **104,** 294–295.

YAU, L., PINSK, M. & ZAHRADKA, P. (1996). Inhibition of RNA synthesis by bradykinin involves both the B1 and B2 receptor subtypes. *Arch. Biochem. Biophys.,* **328,** 115–121.

YELLON, D.M., ALKHULAIFI, A.M. & PUGSLEY, W.B. (1993). Preconditioning the human myocardium. *Lancet,* **342,** 276–277.

ZICHE, M., MORBIDELLI, L. & DONNINI, S. (1996). Angiogenesis. *Exp. Nephrol.,* **4,** 1–14.

ZICHE, M., MORBIDELLI, L., MASINI, E., AMERINI, S., GRANGER, H.J., MAGGI, C.A., GEPPETTI, P. & LEDDA, F. (1994). Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J. Clin. Invest.,* **94,** 2036–2044.

ZICHE, M., MORBIDELLI, L., PARENTI, A., AMERINI, S., GRANGER, H.J. & MAGGI, C.A. (1993). Substance P increases cyclic GMP levels on coronary postcapillary venular endothelial cells. *Life Sci.,* **53,** 1105–1112.

ZICHE, M., PARENTI, A., LEDDA, F., DELL'ERA, P., GRANGER, H.J., MAGGI, C.A. & PRESTA, M. (1997). Nitric oxide promotes proliferation and plasminogen activator production by coronary venular endothelium through endogenous bFGF. *Circ. Res.,* **80,** 845–852.

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