Pulse exposure to protein synthesis inhibitors enhances vascular responses to des-Arg⁹-bradykinin: possible role of interleukin-1

Denis deBlois, Johanne Bouthillier & ¹François Marceau

Centre de recherche de l'Université Laval, Hôtel-Dieu de Québec, 11, côte du Palais, Québec, QUE, Canada G1R 2J6

1 The modulation of the spontaneous increase in contractile responses to des-Arg⁹-bradykinin (des-Arg⁹-BK) of rabbit aortic strips incubated *in vitro* was studied. Rapid hypotensive responses to exogenous kinins were also measured in rabbits anaesthetized 5 h following pretreatment.

2 Continuous exposure to the protein synthesis inhibitors cycloheximide (71 μ M) or anisomycin (3.8 μ M) profoundly inhibited the sensitization to des-Arg⁹-BK in incubated aortic strips. However, temporary (3 h) inhibition of protein synthesis *in vitro* followed by further incubation (3 h) of tissues without inhibitor, paradoxically enhanced both the maximal contractile responses to des-Arg⁹-BK (1.7 μ M) and the apparent affinity of the kinin without affecting contractions to noradrenaline (NA, 100 nM) at 6.5 h.

3 The stimulatory activity of the short treatment (pulse) with cycloheximide was abolished in the presence of dexamethasone sodium phosphate (100 μ M throughout the incubation). The function of receptors for kinins did not appear to be altered directly by the steroid treatment.

4 Interleukin-1 β (IL-1 β), applied at low concentrations (100–250 pg ml⁻¹) on aortic strips between 3 h and 6.5 h of incubation time, mimicked the selective stimulatory effect of the cycloheximide pulse on responses to des-Arg⁹-BK. Higher concentrations of IL-1 β (0.5–5 ng ml⁻¹) did not further amplify the responses to des-Arg⁹-BK but decreased the contractile responses to NA.

5 The modulation by IL-1 β of vascular sensitivity to des-Arg⁹-BK and to NA was prevented by blockade of protein synthesis.

6 The induction *in vivo* by IL-1 β (5 μ g kg⁻¹) or by cycloheximide (10 mg kg⁻¹) of cardiovascular responsiveness to des-Arg⁹-BK was demonstrated with a blood pressure assay of exogenous kinins or with tissues isolated *ex vivo* 5 h after pretreatment of animals. Evidence of active disposition of cycloheximide *in vivo* was also obtained.

7 We propose the production of endogenous IL-1 as a possible mechanism for the enhancement of responsiveness to des-Arg⁹-BK observed in tissues pulsed with a protein synthesis inhibitor and for the inducing effect of cycloheximide or *E. coli* lipopolysaccharide *in vivo*. These results suggest that effects mediated by the BK₁ receptor for kinins are potentially present in pathological conditions associated with IL-1 production.

Keywords: Des-Arg⁹-bradykinin, biological activity; rabbit aortic strips; sensitization to kinins, modulation; cycloheximide, vascular effects; interleukin-1

Introduction

The generation of vasoactive kinins, such as bradykinin (BK), from circulating blood kininogen is characteristic of the early phase of inflammatory reactions following tissue injury or Gram negative infection (Marceau *et al.*, 1983; Mason *et al.*, 1970). The vascular effects of BK include vasodilatation and increased vascular permeability (Elliot *et al.*, 1960). These effects are thought to be mediated by BK₂ receptors for kinins which are prevalent in vascular tissues under non-pathological conditions (Regoli & Barabé, 1980; Marceau *et al.*, 1983). BK is rapidly converted to its relatively stable metabolite without the C-terminal arginine residue, des-Arg⁹-BK, by kininase I present in plasma and exudates (Erdös & Sloane, 1962; Proud *et al.*, 1987). This conversion is often considered as an inactivation because, in contrast to BK, the affinity of des-Arg⁹-BK for BK₂ receptors is very low (Regoli & Barabé, 1980).

However, des-Arg⁹-BK may become biologically active following the appearance of a distinct type of receptor, termed BK₁, selective for kinin fragments without the C-terminal arginine residue (Regoli & Barabé, 1980). The rabbit aorta incubated in Krebs solution is an *in vitro* model of vascular sensitization specific to kinins such as des-Arg⁹-BK (Regoli *et al.*, 1977). The vascular tissue, which is initially insensitive

either to BK or to des-Arg⁹-BK, shows a selective increase in contractile responses to kinins as a function of incubation time *in vitro* (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988). In this system, the effect of kinins is thought to be mediated through the BK₁ receptor for kinins because des-Arg⁹-BK is ten times more potent than BK and because the effects of all kinins are blocked by the selective BK₁ receptor antagonist [Leu⁸]-des-Arg⁹-BK (for review see Regoli & Barabé, 1980). The sensitization process occurs even in endothelium-denuded aortae (Bouthillier *et al.*, 1987). However, it is dependent on the *de novo* synthesis of protein as it is blocked by continuous application of the protein synthesis inhibitor (PSI) cycloheximide (Bouthillier *et al.*, 1987). Finally, the increase in responsiveness to kinins in the incubated rabbit aorta is almost completely inhibited by the anti-inflammatory steroid dexamethasone sodium phosphate (DSP) (deBlois *et al.*, 1988).

Other smooth muscle preparations in which spontaneous sensitization to the contractile action of des-Arg⁹-BK has been observed include strips of human colon (Couture *et al.*, 1981), rat isolated duodenum (Boschcov *et al.*, 1984) and urinary bladder (Marceau *et al.*, 1980), and rabbit basilar artery (Whalley *et al.*, 1983). In addition, des-Arg⁹-BK elicits vasorelaxant responses mediated by cyclo-oxygenase products in the rabbit coeliac artery (Ritter *et al.*, 1989) and mesenteric artery (Churchill & Ward, 1986; deBlois & Marceau, 1987). In the rabbit mesenteric artery, the increase in responsiveness to

¹ Author for correspondence.

des-Arg⁹-BK is cycloheximide-sensitive as in tissues contracted by the kinin, and it is reduced but not abolished by the complete removal of the endothelium (deBlois *et al.*, 1987).

Selective induction of cardiovascular sensitivity to des-Arg⁹-BK is also observed *in vivo* in rabbits injected with *E. coli* lipopolysaccharide (LPS) (Regoli *et al.*, 1981; deBlois *et al.*, 1989). Unlike control animals, where it has no apparent effect, an intra-arterial bolus of des-Arg⁹-BK causes a rapid, transient hypotensive response in anaesthetized rabbits 5 h after pretreatment with LPS. In hearts obtained from LPS-treated animals and perfused *ex vivo* at constant flow, des-Arg⁹-BK elicits a BK₁ receptor-mediated decrease in peripheral vascular resistance (Regoli *et al.*, 1981). The precise mechanism of the hypotensive effect of des-Arg⁹-BK in the rabbit is not known. Kinins may act on endothelial BK₁ receptors to release vasodilator agents such as endothelium-derived relaxing factor or prostacyclin as shown for cultured bovine endothelial cells (D'Orleans-Juste *et al.*, 1989). Alternatively, des-Arg⁹-BK may act on other cell types (e.g. smooth muscle) to produce vasorelaxation as suggested by the significant residual responses to des-Arg⁹-BK observed in endothelium-denuded mesenteric arteries (deBlois & Marceau, 1987).

Rabbits injected with LPS produce interleukin-1 β (IL-1 β) *in vivo* (Cannon *et al.*, 1989). Cultured vascular smooth muscle and endothelial cells also produce IL-1 in response to LPS (Libby *et al.*, 1986a,b). Moreover, LPS and IL-1 β are potent stimulants of the sensitization to des-Arg⁹-BK in rabbit aortae incubated *in vitro* (Bouthillier *et al.*, 1987; deBlois *et al.*, 1988). This raises the possibility that the vascular sensitization to des-Arg⁹-BK may be associated with the endogenous production of IL-1. In addition to LPS, inhibition of protein synthesis also induces the production of IL-1 mRNA in vascular smooth muscle (Warner *et al.*, 1987) and endothelial cells (Libby *et al.*, 1986a). Furthermore, removal of the PSI may lead to the increased production of protein(s) corresponding to the accumulated mRNA(s) (Warner & Libby, 1989). Thus, we speculated that a transient inhibition of protein synthesis might increase vascular responsiveness to des-Arg⁹-BK. The experiments described here provide evidence for the association between IL-1 and vascular sensitivity to des-Arg⁹-BK. We found that vascular responses to des-Arg⁹-BK are enhanced by a transient inhibition of protein synthesis *in vitro* or *in vivo*, and by IL-1 β injected *in vivo* into rabbits. The induction by IL-1 of BK₁ receptors for relatively stable metabolites of kinins may accentuate and prolong the hypotensive effect of kinins in pathologies associated with a dramatic fall in blood pressure, such as the septic shock (Wilson *et al.*, 1989).

Methods

Isolated blood vessels

The thoracic aorta was isolated from New Zealand White rabbits of either sex (1.2 to 1.8 kg). The vessels were prepared, suspended under a basal tension of 2 g in 5 ml organ chambers containing Krebs solution, and their responses to agents were recorded as previously described (Bouthillier *et al.*, 1987). The level of responsiveness to des-Arg⁹-BK was monitored by applying the kinin fragment at a concentration of 1.7 μ M, which is approximately the EC₉₅ (Regoli & Barabé, 1980). The upper panel of Figure 1 represents the schematic diagram for the application of vasoactive agents during the incubation of the smooth muscle preparation. Des-Arg⁹-BK was routinely applied 1, 3 and 6 h after the beginning of the tissue incubation, and noradrenaline (NA, 100 nM) after 1.5 and 6.5 h of incubation. The repeated stimulations with des-Arg⁹-BK were done in order to monitor the progressive increase of responsiveness of aortic strips to the kinin metabolite. NA was applied to monitor the response to an unrelated contractile agent. Tissues were allowed to respond for 10 min. At the end

of this period, the vasoactive agents were removed by washing the tissues twice with fresh Krebs solution. The maximal level of contraction observed during the 10 min observation period is presented and is expressed in g of isometric tension. The longest period between washings with fresh Krebs solution was 90 min.

Pharmacological modulation of *in vitro* sensitization to des-Arg⁹-bradykinin

The lower panel of Figure 1 is a summary of the various types of treatment applied on the rabbit aortic strips during the *in vitro* incubation. Tissues were exposed to a PSI (solid bars), namely cycloheximide (71 μ M) or anisomycin (3.8 μ M), for various periods of time *in vitro*: (a) for the first 1 h, (b) for the first 3 h, or (c) for the whole incubation period. In strips temporarily treated (pulsed) with a PSI, the fluid bathing the tissues was changed every hour after the end of the exposure to the inhibitor. This protocol was followed to investigate the possible stimulating effect of a temporary blockade of protein synthesis on the vascular sensitization to des-Arg⁹-BK. In some experiments, incubated strips were exposed continuously to the inhibitory steroid DSP (100 μ M, hatched bars) which was applied (d) alone or (e) in combination with a PSI for the first 3 h. Some strips were treated for the last 3.5 h of incubation with (f) DSP (100 μ M) or with (g) IL-1 β (0.1–5 ng ml⁻¹, open bars). IL-1 β (5 ng ml⁻¹) was also applied for the first 3 h (h) alone or (i) in combination with a high concentration of a PSI (anisomycin, 38 μ M).

In vivo sensitization to des-Arg⁹-bradykinin

Five hours prior to any other experimental procedure, rabbits were given an i.v. injection of one of the following agents: cycloheximide (1 or 10 mg kg⁻¹), *E. coli* LPS (25 μ g kg⁻¹), IL-1 β (5 μ g kg⁻¹) or tumor necrosis factor α (TNF α , 5 μ g kg⁻¹). Control animals received 1 ml of physiological saline (1 ml) or bovine serum albumin (BSA, 1 mg kg⁻¹) dissolved in physiological saline which was the vehicle for IL-1 β and TNF α .

Two experimental approaches were used to monitor the vascular sensitization to des-Arg⁹-BK that occurred *in vivo* following the pretreatments. First, vascular responses to des-Arg⁹-BK were measured *ex vivo* in aortic strips isolated from pretreated rabbits. Strips were prepared and suspended in organ chambers as described above. The sensitization to des-Arg⁹-BK occurs spontaneously *in vitro* in aortae isolated from normal rabbits (Bouthillier *et al.*, 1987). However, it is both absent at 1 h and repressed by continuous exposure to cycloheximide. Thus, the following procedure was adopted to measure specifically the degree of sensitization to des-Arg⁹-BK occurring *in vivo* without interference from the *in vitro* sensitization. The aortic strips were routinely incubated in the presence of cycloheximide (71 μ M) and stimulated with des-Arg⁹-BK (1.7 μ M) at 1 h. The contractile response to NA (100 nM) at 1.5 h was also measured.

Second, rapid hypotensive responses to exogenous kinins were measured in rabbits anaesthetized 5 h following pretreatment. Rabbits were anaesthetized with lignocaine and sodium pentobarbitone and ventilated as described previously (Regoli *et al.*, 1981). Immediate blood pressure changes in response to intra-arterial injection of BK or of des-Arg⁹-BK were recorded from the left carotid artery as described by Bouthillier *et al.* (1987).

In a separate set of experiments, rabbits were injected with the angiotensin converting enzyme inhibitor captopril (5 or 10 mg kg⁻¹) or enalapril (5 or 10 mg kg⁻¹) and anaesthetized 20 h later for blood pressure measurements. This longer delay between the pretreatments and the anaesthesia was chosen to reproduce an experimental procedure previously described (Nwator & Whalley, 1989) which reportedly induced in 20 h a state of cardiovascular sensitivity to des-Arg⁹-BK. In addition,

Figure 1 Upper panel: schematic diagram for the application of vasoactive agents during the incubation of the rabbit aortic strips. Des-Arg⁹-bradykinin (des-Arg⁹-BK, 1.7 μ M) was routinely applied 1, 3 and 6 h after the beginning of the tissue incubation, and noradrenaline (NA, 100 nM) after 1.5 and 6.5 h of incubation. Tissues were allowed to respond for 10 min. At the end of this period, the vasoactive agents were removed by washing the tissues twice with fresh Krebs solution. Lower panel: summary of the various types of treatment applied on the rabbit aortic strips during the *in vitro* incubation. Tissues are exposed to a protein synthesis inhibitor (PSI, solid bars), namely cycloheximide (71 μ M) or anisomycin (3.8 μ M), for various periods of time *in vitro*: (a) for the first 1 h, (b) for the first 3 h, or (c) for the whole incubation period. In some experiments, incubated strips were exposed continuously to the inhibitory steroid dexamethasone sodium phosphate (DSP, 100 μ M, hatched bars) which was applied (d) alone or (e) in combination with a PSI for the first 3 h. Some strips were treated for the last 3.5 h of incubation with (f) DSP (100 μ M) or with (g) interleukin-1 β (IL-1 β , open bars) at increasing concentrations (0.1–5 ng ml⁻¹). IL-1 β (5 ng ml⁻¹) was also applied for the first 3 h (h) alone or (i) in combination with a high concentration of PSI (anisomycin, 38 μ M).

a group of rabbits was anaesthetized for blood pressure measurements 5 h after an i.v. injection of captopril (5 mg kg⁻¹).

Time course of protein synthesis inhibition by cycloheximide *in vivo*

The transient nature of vascular cells exposure to cycloheximide *in vivo* was investigated by measuring the inhibition of [³⁵S]-methionine incorporation into cultured fibroblasts by plasma from a cycloheximide-treated rabbit. At various intervals following pretreatment, a rabbit injected with cycloheximide (10 mg kg⁻¹) was bled through the central ear artery into tubes containing a final heparin concentration of 18 u ml⁻¹. The plasma was collected by centrifugation at 4°C and stored frozen at -20°C. Rabbit dermal fibroblasts were derived from subcutaneous explants, cultured in Dulbecco's Modified Eagle Medium (DMEM, from Gibco, Grand Island, NY, U.S.A.) supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 2% non-essential amino-acids, 100 u ml⁻¹ penicillin and 100 u ml⁻¹ streptomycin as described (Marceau & Tremblay, 1986; all reagents from Gibco). Cells (1.0 × 10⁵) were seeded in each well of a 12-well plate (Linbro, Flow Labs, Mclean, VA, U.S.A.; well diameter, 24 mm) and further cultured for 2 days. Then the culture medium was replaced with DMEM containing 5% FCS, 5% rabbit plasma from the cycloheximide-treated animal and [³⁵S]-methionine

(10 μ Ci ml⁻¹; specific activity 200 mCi mmol⁻¹, Amersham, Oakville, Canada). At the end of the incubation, the cells were washed extensively with phosphate buffered (78 mM, pH 7.4) saline and solubilized in 1 ml NaOH (0.15 N). Scintillation cocktail (Scintiverse, Fisher Scientific, Fairlawn, NJ, U.S.A.) was added and the radioactivity incorporated into the cells was measured.

Drugs

Cycloheximide, anisomycin and (-)-noradrenaline were purchased from Sigma (St Louis, MO, U.S.A.). Bradykinin and des-Arg⁹-BK were from Bachem (Torrance, CA, U.S.A.). Dexamethasone sodium phosphate was from Sabex (Montréal, Québec, Canada), heparin sodium U.S.P. was from Organon (Toronto, Ontario, Canada). Lipopolysaccharide from *E. coli*, 0111:B4, was from DIFCO Lab. (Detroit, MI, U.S.A.). Interleukin-1 β (recombinant, human sequence) was a gift from Biogen S.A. (Geneva, Switzerland) and tumor necrosis factor α (recombinant, human sequence) a gift from Genetics Institute (Boston, MA, U.S.A.). Pentobarbitone sodium was from Abbot Laboratories (Toronto, Ontario, Canada) and lignocaine hydrochloride from Astra (Mississauga, Ontario, Canada). Captopril and enalapril were gifts from the Squibb and Merck companies, respectively. Endotoxin content of the cycloheximide and IL-1 β solutions

that were injected *in vivo* in some experiments, was verified by the *Limulus* amoebocyte lysate assay (Sigma).

Statistical analysis

Results are expressed as means $\pm$ standard errors (s.e.) of the mean. In most *in vitro* protocols, responses obtained in treated tissues were matched with those from paired controls from the same rabbit; results were then analysed by means of one way analysis of variance (ANOVA) followed by Student's *t* test for paired samples in order to minimize the effect of inter-animal variation. ANOVA followed by Dunnett's test was used when more than three comparisons were made between treated groups or with the control group (Tallarida & Murray, 1987).

Results

Effect of protein synthesis inhibitors on responses to des-Arg⁹-bradykinin *in vitro*

Initially insensitive to a near maximal concentration of des-Arg⁹-BK (1.7 μ M), the rabbit isolated aorta selectively increased its contractile response to the kinin as a function of incubation time *in vitro* (Figure 2a). The effect of PSIs on this sensitization process varied according to the time schedule of exposure *in vitro* (Figure 2, typical tracings; Figure 3a and b, statistical analyses). Whereas application of cycloheximide (71 μ M) throughout the incubation period completely inhibited the development of vascular sensitivity to des-Arg⁹-BK, a short application of the PSI during the first hour significantly increased the level of maximal response to des-Arg⁹-BK recorded after 6 h of incubation. Tissues exposed to cyclo-

Figure 2 Contractile effect of des-Arg⁹-bradykinin (des-Arg⁹-BK, 1.7 μ M) and of noradrenaline (NA, 100 nM) on the rabbit isolated aorta as a function of incubation time *in vitro*. Three aortic strips isolated from the same animal were stimulated with des-Arg⁹-BK for 10 min after 1, 3 and 6 h of incubation and with NA after 1.5 and 6.5 h of incubation. Exposure to NA was ended after the peak contractile response was reached. Cycloheximide (71 μ M) was applied to paired strips from the same aorta (b) for the first 3 h of incubation or (c) was present continuously in the bathing fluid. One tissue (a) was left untreated as a control for spontaneous sensitization. Ordinate scale: isometric contraction in g; abscissa scale: time in min. Closed symbols indicate the time of the application of the vasoactive agents and open symbols indicate the end of the stimulation period. The spontaneous increase in responses to des-Arg⁹-BK of incubated rabbit aortic strips was selectively amplified following a temporary blockade of protein synthesis. Continuous exposition to cycloheximide abolished the sensitization to the kinin.

Figure 3 Selective increase in responses to des-Arg⁹-bradykinin (des-Arg⁹-BK, 1.7 μ M) of the incubated rabbit aorta transiently exposed to a protein synthesis inhibitor (PSI). Responses to the kinin (a and b) and to noradrenaline (NA, 100 nM, c and d) are shown. The vasoactive agents were applied on tissues for 10 min. Adjacent hatched and solid columns refer to measurements in the same set of tissue at different times during the incubation: des-Arg⁹-BK was applied after 3 h (hatched columns) and 6 h (solid columns) and NA after 1.5 h (hatched columns) and 6.5 h (solid columns). Some tissues were exposed to cycloheximide (71 μ M, $n = 8-10$, a and c) or anisomycin (3.8 μ M, $n = 5-7$, b and d) for the first 1 or 3 h of incubation. Another set of tissues were exposed continuously (0-6.5 h) to the PSI. Vertical lines represent s.e. of the mean. Statistically significant differences between responses of treated and control tissues at 3 h or at 6 h were determined by one way analysis of variance followed by Student's *t* test: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

heximide for the first 3 h of incubation did not respond to des-Arg⁹-BK at the end of this period. However, when the same tissues were incubated for an additional 3 h without cycloheximide and then stimulated with the kinin, a markedly higher level of contraction was observed (Figures 2b and 3a). Contractions at 6 h were significantly greater following a long exposure (first 3 h of incubation) than a short exposure (first 1 h) to cycloheximide. Anisomycin, a PSI structurally unrelated to cycloheximide, behaved similarly: it prevented or increased the development of responses to des-Arg⁹-BK depending whether it was applied continuously or transiently

to the incubated rabbit aorta (Figure 3b). Contractions to NA (100 nM) were not affected by any of the treatments (Figure 3c and d).

Concentration-effect curves of des-Arg⁹-bradykinin

Complete cumulative concentration-effect curves for des-Arg⁹-BK were established at 6 h as previously described (Regoli *et al.*, 1978) in tissues exposed to cycloheximide for the first 3 h of incubation. As already shown in Figure 3a, this treatment resulted in an increase in the maximal response of aortic strips to des-Arg⁹-BK (Figure 4a). In addition, the concentration-effect curve expressed as a percentage of the maximal response was shifted to the left in comparison with the paired control curve (Figure 4b).

Inhibition by dexamethasone of the stimulant effect of cycloheximide in vitro

Continuous exposure of aortic strips to DSP was shown to inhibit specifically the sensitization to des-Arg⁹-BK even in the presence of known stimulants of the sensitization process (deBlois *et al.*, 1988). We investigated whether the stimulating effect of cycloheximide in this system was sensitive to inhibition by DSP (100 μ M). The stimulant effect of the cycloheximide pulse was virtually abolished by the steroid (Figure 5a). Moreover, the two groups of tissues incubated in the presence of DSP had similar levels of responsiveness to des-Arg⁹-BK at 6 h. We investigated the possibility that a steroid-induced protein could inhibit the contractile response to des-Arg⁹-BK by acting on the receptor or at a post-receptor level. In tissues responsive to des-Arg⁹-BK at 3 h, a subsequent treatment with DSP from 3 h to 6.5 h did not reduce responsiveness to des-Arg⁹-BK at 6 h as compared to 3 h in the same tissue (Figure 5b). This suggests that the receptor function was not altered directly by the steroid treatment.

Figure 4 Concentration-effect curves for des-Arg⁹-bradykinin on (○) control rabbit aortic strips or on tissues exposed to cycloheximide (71 μ M) for the first 3 h of incubation (●). The points represent the mean of 4 determinations made after 6 h of incubation and vertical lines show s.e. mean. The same data are presented (a) as g of contraction or (b) as a % of the maximal contraction. The maximal response to the kinin was increased and the half-maximal concentration was lower in tissues pulsed with cycloheximide as compared to control.

Figure 5 Effect of the glucocorticoid, dexamethasone sodium phosphate (DSP, 100 μ M) on the response to des-Arg⁹-bradykinin (des-Arg⁹-BK, 1.7 μ M) of incubated strips of rabbit aorta. In (a) paired tissues were treated with DSP throughout the incubation period (0-6.5 h) or with cycloheximide (CHX, 71 μ M) for the first 3 h (0-3 h) or with both treatments combined. One group of tissues was left untreated as a control for the spontaneous increase in responsiveness to des-Arg⁹-BK. Responses to the kinin at 6 h are shown as the mean of 6 determinations; s.e. shown by vertical bars. DSP markedly inhibited the increase in responses to des-Arg⁹-BK of rabbit aortic strips even in tissues exposed temporarily to cycloheximide. Student's *t* test for paired samples was used to compare tissues treated with DSP to the corresponding tissue incubated without the steroid. In (b), tissues were incubated with DSP (100 μ M) throughout the incubation period (0-6.5 h), for the last 3.5 h (3-6.5 h) or without the steroid (control). Responses to the kinin in the same set of tissues at 3 h (hatched columns) and 6 h (adjacent solid columns) are shown as the mean of 5 determinations with s.e. shown by vertical bars. In tissues exposed to DSP for the last 3.5 h, the steroid did not reduce the magnitude of the response at 6 h as compared to 3 h. Student's *t* test for paired samples was used to compare the levels of response to des-Arg⁹-BK at 3 h and 6 h within each group of tissues. Responses to noradrenaline were not affected by any of those treatments (not shown). Levels of significance: * *P* < 0.05; ** *P* < 0.01.

Effect of interleukin-1 β on tissue responsiveness to des-Arg⁹-bradykinin

To test whether exogenous IL-1 β could reproduce the stimulatory effect of cycloheximide on the responses to des-Arg⁹-BK in the incubated rabbit aorta, the cytokine was added at various concentrations to the bathing fluid for the last 3.5 h of incubation (3-6.5 h). This schedule of application was chosen to reproduce the putative production of endogenous IL-1 by cells in the vascular wall exposed to cycloheximide for the first 3 h of incubation. Application of IL-1 β at low concentrations (100-250 pg ml⁻¹) for the last 3.5 h of incubation had a selective stimulatory effect on the responses to des-Arg⁹-BK of

aortic strips (Figure 6a and b). Tissues exposed to IL-1 β at these concentrations had a level of response to the kinin (at 6 h) or to NA (at 6.5 h) that was not significantly different from those of tissues exposed to cycloheximide for the first 3 h. In contrast, tissues left untreated as controls for spontaneous sensitization had significantly weaker contractile responses to des-Arg⁹-BK as indicated by ANOVA and Dunnett's test. At higher concentrations of IL-1 β (0.5–5 ng ml⁻¹), the level of responses to the kinin was not further enhanced but the level of contractions in response to NA was significantly reduced (82% inhibition in the presence of 5 ng ml⁻¹ IL-1 β).

Continuous exposure to a high concentration of the PSI anisomycin (38 μ M) prevented the sensitization to des-Arg⁹-BK even in tissues incubated with IL-1 β (5 ng ml⁻¹; Figure 7a) suggesting that the synthesis of one or more proteins different from IL-1 is necessary for the sensitization to the kinin. In addition, the desensitization to NA in tissues exposed to the IL-1 β was prevented by blockade of protein synthesis (Figure 7b).

In vivo induction of vascular sensitivity to des-Arg⁹-bradykinin

Since cycloheximide was shown to enhance the spontaneous development of responsiveness to des-Arg⁹-BK *in vitro*, we administered the agent to rabbits in order to verify whether it could induce *in vivo* a state of vascular responsiveness to the kinin metabolite. LPS, a known inducer of cardiovascular

Figure 6 Effects of a temporary exposure to cycloheximide or to interleukin-1 β on the contractile response of rabbit aortic strips to des-Arg⁹-bradykinin (des-Arg⁹-BK, 1.7 μ M) and to noradrenaline (100 nM). Strips were exposed to cycloheximide (CHX, 71 μ M) for the first 3 h of incubation (0–3 h) or to interleukin-1 β (IL-1 β) at various concentrations for the last 3.5 h (IL-1 β , 3–6.5 h). Responses to the kinin at 6 h are shown as the mean of 7–19 determinations; vertical bars show s.e. IL-1 β , applied at low concentrations for the last 3.5 h of incubation, mimicked the selective stimulatory effect of the pulse of cycloheximide on the contractile response to des-Arg⁹-BK. The control and IL-1 β -treated groups were compared to the cycloheximide-treated group by use of one-way analysis of variance followed by Dunnett's test. Levels of significance: ** $P < 0.01$; *** $P < 0.001$.

Figure 7 Inhibitory effect of anisomycin in the presence of interleukin-1 β (IL-1 β) on the sensitization of the incubated rabbit aorta to des-Arg⁹-bradykinin (des-Arg⁹-BK). Paired aortic strips were incubated in the presence of a high concentration of anisomycin (38 μ M, 0–6.5 h) or IL-1 β (5 ng ml⁻¹, 0–3 h) or a combination of the two agents. One tissue was left untreated as a control for spontaneous sensitization to the kinin. Responses (a) to des-Arg⁹-BK (1.7 μ M) at 6 h and (b) to noradrenaline (NA, 100 nM) at 6.5 h are shown as the mean of 5 determinations; vertical bars show s.e. Anisomycin (AN) prevented the sensitization to des-Arg⁹-BK in tissues incubated with or without IL-1 β as well as the desensitization to NA in tissues exposed to the cytokine. In (a), strips exposed to anisomycin were compared to the corresponding tissue incubated without the inhibitor by use of Student's *t* test for paired samples. In (b), Dunnett's test was used to compare all groups to the control group. Levels of significance: ** $P < 0.01$; *** $P < 0.001$.

sensitivity to des-Arg⁹-BK (Regoli *et al.*, 1981) and IL-1 β , the mRNA of which is induced in cultured vascular cells by LPS or by cycloheximide (Warner *et al.*, 1987; Libby *et al.*, 1986b), were also injected separately *in vivo*.

Thoracic aortae from rabbits treated *in vivo* with cycloheximide (10 mg kg⁻¹ i.v., 5 h before isolation) had a significantly higher level of contraction in response to des-Arg⁹-BK after a short period (1 h) of incubation *in vitro* than tissues isolated from saline-treated rabbits (Figure 8a). The effect of cycloheximide was dose-dependent and did not reach statistical significance at the lower dose tested (1 mg kg⁻¹). LPS (25 μ g kg⁻¹) was a more potent *in vivo* inducer of vascular sensitivity to des-Arg⁹-BK than cycloheximide. The pro-inflammatory cytokine IL-1 β (5 μ g kg⁻¹) was also an inducer *in vivo* whereas its BSA vehicle (1 mg kg⁻¹) was not. None of these pretreatments affected the *in vitro* response to NA (100 nM) recorded at 1.5 h (Figure 8b). Only 1 ng of endotoxin per mg of cycloheximide was detected in the solution of inhibitor administered *in vivo* by the *Limulus* amoebocyte lysate assay. The solution of IL-1 β contained no detectable endotoxin by this assay (detection limit: 10 pg ml⁻¹).

The induction *in vivo* of cardiovascular sensitivity to des-Arg⁹-BK was also demonstrated by a blood pressure assay for exogenous kinins. Rabbits injected with cycloheximide (10 mg kg⁻¹), IL-1 β (5 μ g kg⁻¹) or LPS (25 μ g kg⁻¹), but not

Figure 8 Selective induction *in vivo* of vascular responsiveness to des-Arg⁹-bradykinin (des-Arg⁹-BK) by cycloheximide (CHX, 10 mg kg⁻¹), *E. coli* lipopolysaccharide (LPS, 25 μg kg⁻¹) or interleukin-1β (IL-1β, 5 μg kg⁻¹) as assessed *in vitro* by the contractile effects of (a) des-Arg⁹-BK (1.7 μM) after 1 h of incubation or (b) noradrenaline (100 nM) at 1.5 h in strips of rabbit aorta isolated 5 h after the i.v. injection of the agent. Aortic tissues were incubated *in vitro* in the presence of cycloheximide (71 μM). Vertical bars represent s.e.mean of 8 determinations. Values were compared to control (saline) by use of one way analysis of variance followed by Dunnett's test, except for IL-1β which was compared to the bovine serum albumin (BSA)-treated group by the use of Student's *t* test for unpaired samples. Levels of significance: * *P* < 0.05; ** *P* < 0.01.

with the saline or the BSA (1 mg kg⁻¹) vehicles, developed significant hypotensive responses to exogenous des-Arg⁹-BK given as an intra-arterial bolus 5 h after the pretreatment (Table 1). LPS was the most potent inducer at doses tested. Rabbits pretreated with TNFα (5 μg kg⁻¹, *n* = 2), a pro-inflammatory cytokine which is induced by LPS in cultured smooth muscle cells (Warner & Libby, 1989), did not respond to exogenous des-Arg⁹-BK (data not shown). Responses to exogenous bradykinin were present in the saline-treated group

(25 ng: 5.1 ± 0.4 mmHg; 100 ng: 13.8 ± 3.1 mmHg; 250 ng: 20.1 ± 1.5 mmHg) and were not affected by any of the pretreatments (data not shown).

In addition, it has been reported that captopril (5 mg kg⁻¹) or enalapril (5 mg kg⁻¹), which are widely used inhibitors of the angiotensin converting enzyme, could induce a state of cardiovascular responsiveness to des-Arg⁹-BK when administered i.v. to rabbits 20 h before anaesthesia (Nwator & Whalley, 1989). These results could not be reproduced in our laboratory even with a double dose of the agent (Table 1). Moreover, responses to exogenous des-Arg⁹-BK were also monitored in rabbits that had received captopril (5 mg kg⁻¹) 5 h only before anaesthesia. This experiment was undertaken because of a possible 'fading away' of the vascular sensitivity during the 20 h experimental period. No significant response to exogenous des-Arg⁹-BK could be observed under these conditions (Table 1).

Time course of protein synthesis inhibition by i.v. cycloheximide

[³⁵S]-methionine incorporation into cultured fibroblasts was inhibited by 69% following incubation with 5% (v/v) plasma sampled 5 min after i.v. injection of cycloheximide (10 mg kg⁻¹) (Table 2). The inhibition of [³⁵S]-methionine incorporation was less extensive with plasmas obtained later

Table 2 [³⁵S]-methionine incorporation into cultured rabbit dermal fibroblasts exposed for 6 h to rabbit plasma (5% v/v) sampled following i.v. injection with cycloheximide (10 mg kg⁻¹)^a

Time of blood sampling	[³⁵ S]-methionine incorporated ^c (c.p.m. × 10 ⁻²)
Control ^b	708 ± 51
5 min	232 ± 17 ^d
30 min	283 ± 7 ^d
60 min	347 ± 17 ^d
120 min	473 ± 23 ^d
180 min	551 ± 38 ^d
240 min	645 ± 27

^a Rabbit dermal fibroblasts were incubated for 6 h in Dulbecco's Modified Eagle Medium in the presence of 5% FCS (v/v) and 5% plasma sampled at various times following injection of cycloheximide (10 mg kg⁻¹) to a rabbit.

^b Rabbit plasma sampled before cycloheximide injection.

^c Results are expressed as c.p.m. of [³⁵S]-methionine incorporated per 10⁵ cells and represent the mean ± s.e. of quadruplicate estimates.

^d Value significantly different from control (*P* < 0.01 as calculated by Dunnett's test).

Table 1 Effect of des-Arg⁹-bradykinin on the mean arterial blood pressure of anaesthetized rabbits subjected to various treatments *in vivo*

Treatment (dose) ^a	n	Basal blood pressure (mmHg)		Hypotensive effect of des-Arg ⁹ -bradykinin		
		Systolic	Diastolic	0.25 μg	1.0 μg	2.5 μg
Saline (1 ml)	4	116 ± 1	97 ± 1	0 ^b	0	0
Cycloheximide (10 mg kg ⁻¹)	9	109 ± 5	91 ± 4	4 ± 1	8 ± 2*	13 ± 2**
LPS (25 μg kg ⁻¹) ^c	4	105 ± 9	80 ± 8	6 ± 1*	15 ± 5*	19 ± 6*
BSA (1 mg kg ⁻¹)	8	110 ± 3	87 ± 6	0	0	0
IL-1β (5 μg kg ⁻¹)	12	105 ± 4	78 ± 4	3 ± 1***	5 ± 1***	8 ± 1***
Captopril (5 mg kg ⁻¹)	3	113 ± 8	76 ± 1	0	0	0
Captopril (5–10 mg kg ⁻¹) ^d	6	106 ± 6	78 ± 5	0	0	0
Enalapril (5–10 mg kg ⁻¹) ^d	4	109 ± 11	77 ± 5	0	0	0

^a Treatments were in the form of a single i.v. injection 5 h before anaesthesia, except for captopril (5–10 mg kg⁻¹) and for enalapril which were given 20 h before anaesthesia.

^b Fall of mean arterial blood pressure, mmHg. Results are compared with the control group (saline) by use of Dunnett's test, except for IL-1β, which was compared to BSA by use of Student's *t* test. Significance levels are expressed as follows: * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001.

^c Abbreviations used: LPS: lipopolysaccharide; BSA: bovine serum albumin; IL-1β: interleukin-1β.

^d Pool of results obtained with an equal number of animals treated with a single or a double dose of the agent.

on. Plasma sampled 4 h after cycloheximide injection did not significantly inhibit [3 S]-methionine incorporation as compared to control (Table 2). These results suggest that an active disposition of cycloheximide occurs in rabbits *in vivo* within the 5 h experimental period used in this study.

Discussion

Des-Arg⁹-BK is hypotensive in rabbits injected with LPS and a vasodilator in perfused hearts from these treated animals (Regoli *et al.*, 1981). By contrast, it is a vasoconstrictor in the rabbit aortic preparation (Regoli *et al.*, 1981; deBlois *et al.*, 1989). The fall in mean arterial blood pressure in response to des-Arg⁹-BK is probably not functionally related to the contractile response of the aorta. Nevertheless, this tissue was used to study the process of vascular sensitization to des-Arg⁹-BK *in vitro* for three reasons. First, the myotropic response of the rabbit aorta to kinins is mediated exclusively by BK₁ receptors and has been extensively characterized (for review see Regoli & Barabé, 1980). Secondly, the rabbit aorta being insensitive to vasoactive prostaglandins which may interfere with the contractile response (Förstermann *et al.*, 1984), responds to des-Arg⁹-BK with a monophasic contraction (Bouthillier *et al.*, 1987). This makes the sensitization easier to study than, for instance, that occurring in the mesenteric artery in which a contraction is followed by a prostaglandin-mediated relaxation (deBlois & Marceau, 1987). Finally, in rabbits sensitized *in vivo* with LPS, the contractile response of the isolated aorta to des-Arg⁹-BK is a good indicator of the systemic cardiovascular sensitivity to the kinin observed by use of the blood pressure assay (Regoli *et al.*, 1981; deBlois *et al.*, 1989; present results).

The *de novo* synthesis of BK₁ receptors for kinins has been postulated to account for the increased responses to des-Arg⁹-BK observed in smooth muscle preparations incubated *in vitro* and in the cardiovascular system of endotoxaemic rabbits (Regoli *et al.*, 1978; Marceau *et al.*, 1983). Although the precise mechanism of sensitization is still unknown, the dependence on protein synthesis of the *in vitro* process is well established. In the present study, we have documented a paradoxical stimulant effect of PSIs on the development of vascular responses to des-Arg⁹-BK *in vitro* and *in vivo*. We also observed the *in vivo* induction in the rabbit of cardiovascular sensitivity to des-Arg⁹-BK by the pro-inflammatory cytokine IL-1 β . Our results suggest a possible association between the endogenous production of IL-1 and vascular sensitivity to agonists of the BK₁ receptor for kinins.

Stimulating effect of protein synthesis inhibitors *in vitro*

Temporary inhibition of protein synthesis with two structurally unrelated inhibitors, namely cycloheximide and anisomycin, enhanced both the maximal response of the rabbit aorta to des-Arg⁹-BK and the apparent affinity of the kinin. This phenomenon has been reported previously with IL-1 β (deBlois *et al.*, 1988) and epidermal growth factor (EGF; Bouthillier *et al.*, 1987). Although stimulation of EGF production in aortic strips pulsed with cycloheximide *in vitro* cannot be ruled out, it is known that the synthesis of mRNA for IL-1 is induced by cycloheximide in cultured vascular smooth muscle cells (Warner *et al.*, 1987) or endothelial cells (Libby *et al.*, 1986a). Moreover, inhibition of IL-1 synthesis by glucocorticoids (Smith, 1980) may partially account for the inhibitory effect of DSP on responses to des-Arg⁹-BK in tissues pulsed with cycloheximide. The induction but not the subsequent function of receptors for kinins appears to be sensitive to the inhibitory effect of DSP because incubation of kinin-sensitive aortic strips with the glucocorticoid for a period of time sufficient for the synthesis of steroid-induced proteins did not result in a decreased level of response to the kinin.

Low concentrations of IL-1 β mimicked the stimulating effect of cycloheximide on responses to des-Arg⁹-BK. These *in vitro* concentrations were similar to those used to stimulate prostaglandin synthesis in human fibroblasts (Dinarello *et al.*, 1986) and those found in the plasma of children suffering from severe infection (1.5 ng ml^{-1} , Giarardin *et al.*, 1989). This suggests that the production of IL-1 may be a major event regulating the vascular sensitivity to des-Arg⁹-BK.

The inhibition by anisomycin of the spontaneous sensitization to des-Arg⁹-BK that occurs *in vitro* was not reversed by a high concentration of IL-1 β suggesting that the *de novo* synthesis of one or more proteins distinct from IL-1 β is required. This may include *de novo* BK₁ receptor synthesis, the postulated mechanism for the increase in vascular sensitivity to des-Arg⁹-BK (Regoli *et al.*, 1978).

The potent inhibition of contractile responses to NA produced by higher IL-1 β concentrations was prevented by inhibition of protein synthesis. This confirms previous observations made in the rat aorta with α -adrenoceptor agonists (Beasley *et al.*, 1989; McKenna, 1990). A major discrepancy between the rabbit and the rat preparations is the effect of IL-1 β on the apparent contractility of the smooth muscle preparations. Beasley *et al.* (1989) and McKenna (1990) suggested that vascular contractility was depressed non specifically following incubation of rat aortic strips with IL-1 β because it also decreased contractions by KCl. In this study, the enhanced responses to des-Arg⁹-BK in rabbit aortic strips showing a decreased sensitivity to NA suggest that the intrinsic smooth muscle contractility was not depressed.

Induction *in vivo*

As shown with tissues *ex vivo* and with blood pressure measurements in anaesthetized rabbits, a transient inhibition of protein synthesis *in vivo* with i.v. cycloheximide induced an increase in vascular responsiveness to des-Arg⁹-BK. Vascular sensitivity to the kinin metabolite also was induced by injection of IL-1 β ($5 \mu\text{g kg}^{-1}$). IL-1 occurs in the plasma of rabbits injected with LPS (Cannon *et al.*, 1989) and LPS injection produces sensitization to des-Arg⁹-BK *in vivo* (Regoli *et al.*, 1981). Furthermore, cultured vascular cells produce IL-1 in response to LPS (Libby *et al.*, 1986a,b) and IL-1 mRNA in response to cycloheximide (Libby *et al.*, 1986a; Warner *et al.*, 1987). Finally, TNF α ($5 \mu\text{g kg}^{-1}$), a cytokine also induced by LPS but not by cycloheximide in vascular cells, failed to induce a state of vascular sensitivity to des-Arg⁹-BK (not shown). Thus, IL-1 may be the common mediator responsible for the inducing effect of both cycloheximide and LPS on the vascular sensitivity to des-Arg⁹-BK in the rabbit.

It is unlikely that the activity of IL-1 β or cycloheximide *in vivo* is due to a possible contamination by LPS because the *Limulus* amoebocyte lysate assay did not detect any endotoxin in the IL-1 β preparation and only approximately 1 ng endotoxin per mg in the cycloheximide preparation. The stronger *in vivo* inducing effect of cycloheximide (10 mg kg^{-1}) or of LPS ($25 \mu\text{g kg}^{-1}$) as compared to IL-1 β ($5 \mu\text{g kg}^{-1}$) may reflect the ability of these agents to stimulate a sustained endogenous production of cytokines, e.g. IL-1 itself (Dinarello *et al.*, 1987) or IL-2, another *in vitro* stimulant of the sensitization to des-Arg⁹-BK in the rabbit aorta preparation (deBlois *et al.*, 1988). Since IL-1 is frequently synergistic with other cytokines such as IL-2 (Le *et al.*, 1986), the possibility exists that IL-1 action is a necessary but not sufficient step leading to vascular sensitivity to des-Arg⁹-BK. This hypothesis will be tested on rabbit vascular tissues and cells in culture.

Alternatively, it is possible that PSIs stimulate the expression of gene(s) for factors involved in the sensitization to des-Arg⁹-BK but different from IL-1. For example, inhibition of protein synthesis reportedly induces the mRNA for β -interferon (Ringold *et al.*, 1984) and TNF α (Warner & Libby, 1989), and for the nuclear transcription factors *c-myc* (Makino *et al.*, 1984) and *c-fos* (Sariban *et al.*, 1988). Also, a direct tran-

scriptional stimulation of synthesis of membrane receptors for des-Arg⁹-BK (B₁-type) in the presence of PSIs is a possibility that cannot be ruled out at this time.

Nwator & Whalley (1989) reported cardiovascular responses of the BK₁-type in rabbits injected with the angiotensin converting enzyme inhibitors captopril or enalapril 20 h before anaesthesia. The rationale for such an experiment was that the inflammatory side-effects seen with long term use of this group of antihypertensive drugs in man may be related to elevated endogenous kinin and B₁ receptor levels. However, we have been unable to reproduce these results in rabbits injected with a similar or double dose of captopril or enalapril 20 h or 5 h (captopril group) before anaesthesia. The very low baseline blood pressure of anaesthetized animals used in the study of Nwator & Whalley (1989), or traces of endotoxin contaminating the drug preparations (which were administered in appreciable quantity: 5 mg kg⁻¹) may explain the discrepant results.

In summary, we have demonstrated that the spontaneous sensitization to des-Arg⁹-BK of the isolated rabbit aorta was enhanced following short treatments with PSIs. DSP *in vitro* was inhibitory even in tissues pulsed with cycloheximide. The

selective induction *in vivo* by cycloheximide and by IL-1 β of cardiovascular responsiveness to des-Arg⁹-BK was demonstrated using tissues *ex vivo* and a blood pressure assay of exogenous kinins. We propose that the production of endogenous IL-1 is a possible mechanism for the stimulated sensitization to des-Arg⁹-BK observed in tissues pulsed with PSI *in vitro* and for the inducing effect of cycloheximide or LPS *in vivo*. These results suggest that effects mediated by the B₁ kinin receptor may be present in pathological conditions associated with IL-1 production. For example, the sensitization to the hypotensive action of des-Arg⁹-BK observed in rabbits injected with LPS or IL-1 β raises the intriguing possibility that some of the effects of kinins in the septic shock (Wilson *et al.*, 1989) may be mediated by receptors of the BK₁-type.

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