



# Inducible expression of the kinin B<sub>1</sub> receptor in the endotoxemic heart: mechanisms of des-Arg<sup>9</sup>bradykinin-induced coronary vasodilation

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**1** We have investigated the role of kinin B<sub>1</sub> receptor induction in the endotoxemic rat heart and elucidated the mechanisms underlying B<sub>1</sub> receptor-mediated coronary vasodilation. We also investigated the role of these receptors in endotoxin-induced hypotension.

**2** Endotoxin treatment induced cardiac B<sub>1</sub> receptor mRNA expression and promoted a coronary vasodilation response to des-Arg<sup>9</sup>bradykinin (DABK; ED<sub>50</sub> = 149.4 pmol, *n* = 9) *ex vivo* peaking at 6 h. The B<sub>1</sub> receptor antagonist des-Arg<sup>9</sup>[Leu<sup>8</sup>]-BK (DALBK, 30 nM) significantly (*P* < 0.05) inhibited the DABK-induced response (pA<sub>2</sub> = 8.4, *n* = 5) whilst HOE140 (B<sub>2</sub> receptor antagonist, 10 nM) was inactive (*n* = 4).

**3** Removal of the endothelium or infusion with indomethacin (5 μM), but not L-NAME (300 μM) or ODQ (1 μM), inhibited (>85%, *P* < 0.05, *n* = 5) the DABK-induced response. DABK caused a dose-dependent release of the prostacyclin metabolite, 6-keto-PGF<sub>1α</sub> (E<sub>max</sub> = 0.3 ng ml<sup>-1</sup>, *n* = 6).

**4** *In vitro* perfusion of hearts with endotoxin (1 μg ml<sup>-1</sup>, *n* = 6) or interleukin-1β (5 ng ml<sup>-1</sup>, *n* = 6) induced B<sub>1</sub> receptor mRNA expression and promoted a time-dependent vasodilation response to DABK.

**5** Endotoxin treatment (6 h) *in vivo* promoted a hypotensive response to DABK (ED<sub>50</sub> = 29.7 nmol kg<sup>-1</sup>, *n* = 10) which was antagonised by DALBK (3–6 nmol kg<sup>-1</sup> min<sup>-1</sup>, *P* < 0.05, *n* = 7). DALBK (3 nmol kg<sup>-1</sup> min<sup>-1</sup>) and des-Arg<sup>10</sup>HOE140 (B<sub>1</sub> receptor antagonist, 30 nmol kg<sup>-1</sup> min<sup>-1</sup>) produced a 5.3% (*n* = 6, *P* < 0.05) and 8.8% (*n* = 5, *P* < 0.05) reversal, respectively, of endotoxin-induced hypotension.

**6** In summary, we have shown that in endotoxemia activation of B<sub>1</sub> receptors causes coronary vasodilation *via* endothelial prostacyclin release. Additionally, B<sub>1</sub> receptor antagonists partially reversed endotoxin-induced hypotension. Therefore activation of B<sub>1</sub> receptors may have a role to play in the vascular changes associated with endotoxemia.

**Keywords:** Kinin B<sub>1</sub> receptor; endotoxemia; vascular inflammation; rat heart; coronary circulation; nitric oxide; prostanoids

**Abbreviations:** ACh, acetylcholine; BK, bradykinin; CPP, coronary perfusion pressure; DABK, des-Arg<sup>9</sup>bradykinin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; i.p., intraperitoneal; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; L-NAME, N<sup>G</sup>-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MAP, mean arterial pressure; NO, nitric oxide; PCR, polymerase chain reaction; PGI<sub>2</sub>, prostacyclin; SNP, sodium nitroprusside

## Introduction

Kinins act on two receptor types, B<sub>1</sub> and B<sub>2</sub>, classified according to the relative potency of their endogenous agonists (Regoli & Barabe, 1980; Bhoola *et al.*, 1992). While the B<sub>2</sub> receptor is typically activated by bradykinin (BK) and Lys-bradykinin (Lys-BK), the B<sub>1</sub> receptor is selectively sensitive to kinin metabolites without the C-terminal arginine residue, e.g. des-Arg<sup>9</sup>BK (DABK) and Lys-des-Arg<sup>9</sup>BK (Lys-DABK) which show relative selectivity for the rat and human B<sub>1</sub> receptor respectively (Marceau *et al.*, 1998). B<sub>2</sub> receptors are constitutively expressed and mediate many kinin-dependent inflammatory responses including increase in blood flow, vascular permeability and accumulation of inflammatory cells (Regoli & Barabe, 1980; Bhoola *et al.*, 1992). B<sub>1</sub> receptors are not expressed in normal physiology but are induced during vascular inflammation. Knowledge of the contribution of B<sub>1</sub>

receptors to the regulation of vascular tone is of importance since B<sub>1</sub> receptors might replace B<sub>2</sub> receptors during vascular inflammation (Marceau *et al.*, 1998). The selective ligand for B<sub>1</sub> receptors, DABK, normally an inactive metabolite of BK, shows pro-inflammatory activity following an inflammatory insult *in vitro* or *in vivo* (Marceau *et al.*, 1998). B<sub>1</sub> receptor-mediated effects are diverse and include vascular smooth muscle contraction, endothelium dependent vasodilation and inflammatory cell recruitment (for a recent review see, Marceau *et al.*, 1998). However, the mechanisms of B<sub>1</sub> receptor induction and function in the heart is unknown.

B<sub>1</sub> receptors may be involved in certain cardiovascular pathologies such as atheromatous disease (Raidoo *et al.*, 1997) and myocardial ischemia (Bouchard *et al.*, 1998) however, of interest to the present study is the possibility that B<sub>1</sub> receptors are induced and play a role in the vascular inflammatory response to endotoxin. Kinins are elevated during endotoxemia and experimentally endotoxin treatment is associated with activation of the kallikrein-kinin system (DeLa *et al.*, 1993; Nies *et al.*, 1968). Since BK is a potent hypotensive agent in humans and animals (Kontos *et al.*, 1964; Regoli & Barabe,

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1980) it has been suggested that BK acting at constitutive B<sub>2</sub> receptors contributes to the development of hypotension in sepsis. Reports investigating the effects of B<sub>2</sub> receptor selective antagonists in models of endotoxemia nevertheless are conflicting showing both beneficial (Wilson *et al.*, 1989) or no effect (Feletou *et al.*, 1995). However, administration of a B<sub>2</sub> receptor antagonist with moderate efficacy as a B<sub>1</sub> receptor antagonist (CP-0127) inhibits hypotension and improves survival in rats and rabbits challenged with endotoxin (Whalley *et al.*, 1992). This compound was also shown to marginally improve survival in patients with gram-negative sepsis (Fein *et al.*, 1997). It is possible that this marginal beneficial effect is correlated to the marginal efficacy of this compound at B<sub>1</sub> receptors. Moreover, B<sub>1</sub> receptor deficient mice display a blunted hypotensive response to endotoxin (Araujo *et al.*, 1998). It remains unclear however whether the B<sub>1</sub> receptor-mediated hypotensive effect is due to an action at the level of the vasculature or directly on the heart.

The effects of endotoxin on the cardiovascular system, both in terms of the systemic vascular action and the direct effects on the heart are at least partially dependent on enhanced NO production but there is a significant NO-independent component (Ungureanu-Longrois *et al.*, 1995; Meng *et al.*, 1997; Filep *et al.*, 1997). Our hypothesis is that *de novo* induction of B<sub>1</sub> receptors contributes to the NO-independent regulation of coronary tone and hypotension in endotoxemia. To test this hypothesis we investigated whether endotoxin treatment is associated with cardiac B<sub>1</sub> receptor mRNA expression and correlated this with the development of a coronary vasodilation response *ex vivo* and a hypotensive response *in vivo*. We also determined the underlying mechanisms mediating the B<sub>1</sub> receptor induction and coronary vasodilation.

## Methods

### *Animal preparation for in vitro studies*

Experiments were performed according to the 'Guide on the Operation of Animals (Scientific Procedures) Act 1986'. Male Wistar rats (320–370 g Charles River, U.K.) either untreated or treated (1, 2, 3, 6, 14 or 24 h) with lipopolysaccharide (LPS, *E. coli* 0.55:B5, 2.5 mg kg<sup>-1</sup>, i.p.) were heparinized (sodium heparin, 250 iu, i.p.) and killed by cervical dislocation. Hearts were rapidly excised and either frozen in liquid nitrogen for mRNA extraction or placed in ice-cold perfusion solution (constituents below) prior to coronary perfusion pressure (CPP) measurement.

### *Measurement of coronary hemodynamics*

Hearts were cannulated *via* the aorta and perfused retrogradely under constant flow (10 ml min<sup>-1</sup>) using a calibrated roller pump (Gilson, Minipuls-2) with a buffer solution of the following composition (mM): D(+)glucose 11.1, CaCl<sub>2</sub> 1.4, NaCl 118.5, NaHCO<sub>3</sub> 25.0, MgSO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 1.2 and KCl 4.0; gassed with 95% O<sub>2</sub>/5% CO<sub>2</sub>, (pH 7.4) and perfused at 37°C. The CPP in the aortic line was monitored by a Statham Spectramed pressure transducer connected to a chart recorder (Grass 79E). Air temperature was maintained by means of a heated (37°C) water jacket. On establishing a stable CPP (20–30 min following cannulation), bolus doses (10–30 µl) of BK, DABK, sodium nitroprusside (SNP) or acetylcholine were administered *via* the aorta and changes in CPP recorded. Other substances were infused at 0.1 ml min<sup>-1</sup>.

### *Mechanisms of kinin responses*

**Receptor antagonism studies** Control dose-response curves to DABK (10–3000 pmol) or BK (3–1000 pmol) were constructed. Hearts were then perfused with either the B<sub>1</sub> receptor antagonist des-Arg<sup>9</sup>-[Leu<sup>8</sup>]-bradykinin (DALBK, 30 nM) or the B<sub>2</sub> receptor antagonist HOE140 (0.1–10 nM) for 20 min prior to and during construction of a second dose-response curve. These experiments were repeated in hearts from rats pretreated with LPS (2.5 mg kg<sup>-1</sup>; 2, 6, 14 and 24 h). In some experiments CPP was increased in control hearts with endothelin-1 (10 nM, 3 min) prior to the administration of DABK or BK.

Initial studies showed that the induction of the vasodilator response to DABK was maximal in hearts from rats 6 h post LPS-treatment. Therefore the 6 h time point was used in subsequent experiments.

**Determination of endothelium-dependency** The endothelium was removed by saponin infusion (Ellwood & Curtis, 1997); delivered at 20 µg ml<sup>-1</sup> in three cycles of 2 min followed by 2 min buffer, followed by a 20 min control recovery perfusion. Endothelial and vascular smooth muscle function were tested before and after saponin treatment using the endothelium-dependent vasodilator acetylcholine (3 nmol) and the endothelium-independent vasodilator SNP (10 nmol). DABK (1 nmol)-induced responses were determined before and after saponin.

**Involvement of endothelial mediators** In order to investigate involvement of the NO-guanylyl cyclase pathway in mediation of the responses to DABK, dose-response curves were constructed in the absence and presence of either N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME, 300 µM, 20 min pretreatment) to inhibit NO synthase (NOS) or 1H-[1,2,4]oxadiazolo [4,3-*z*]quinoxalin-1-one (ODQ, 1 µM, 20 min pretreatment) to inhibit soluble guanylyl cyclase (Garthwaite *et al.*, 1995). The NO-donor SNP was used to verify the activity of ODQ.

To investigate the involvement of prostanoids, dose-response curves to DABK were constructed in the absence and presence of the cyclooxygenase inhibitor indomethacin (5 µM, 20 min pretreatment). Prostacyclin (PGI<sub>2</sub>) involvement was assessed by measuring the coronary efflux of 6-keto-PGF<sub>1 $\alpha$</sub> , the stable hydrolysis product of PGI<sub>2</sub>, released in response to DABK. Fractions (2 min) were collected immediately before and after administration of DABK. Samples were frozen at -70°C until assay of 6-keto-PGF<sub>1 $\alpha$</sub>  by radioimmunoassay using a commercially available kit (detection limit = 0.06 ng ml<sup>-1</sup>, Amersham Ltd, Bucks, U.K.).

### *In vitro B<sub>1</sub> receptor induction*

Control hearts were perfused *in vitro* with either buffer solution alone (4 h), LPS (1 µg ml<sup>-1</sup>, 4 h) or interleukin-1 $\beta$  (IL-1 $\beta$ , 5 ng ml<sup>-1</sup>, 2 h) and the effects of DABK (1 nmol) tested every 15 min. The concentrations of LPS and IL-1 $\beta$  used were based upon circulating concentrations measured during endotoxemia (Waage *et al.*, 1989; Hanasaki *et al.*, 1997). Responses to BK were also assessed every 30 min during these experiments. At the end of the perfusion hearts were frozen in liquid nitrogen for mRNA extraction.

### *Measurement of blood pressure*

Control or LPS-treated rats were anaesthetized with thiopentone sodium (120 mg kg<sup>-1</sup>, i.p.), the trachea cannulated and

temperature maintained at 37°C with a homeothermic blanket. For measurement of mean arterial pressure (MAP) the right common carotid artery was cannulated and connected *via* a Satham Spectramed pressure transducer to a MacLab4E. The left jugular vein was cannulated for the administration of saline or drugs. To elevate MAP of LPS-treated rats to control levels noradrenaline was infused at a rate of 3–8  $\mu\text{g min}^{-1}$ .

#### Reverse-transcription polymerase chain reaction

Poly(A)<sup>+</sup> mRNA was extracted (Micro-FastTrack kit, Invitrogen, San Diego, CA, U.S.A.) from homogenates of left ventricle, stored at –70°C, from untreated rats, from rats 1, 3, or 6 h post LPS treatment *in vivo* or from hearts perfused *in vitro* with LPS, IL-1 $\beta$ , or buffer solution alone (as described above). Poly(A)<sup>+</sup> mRNA was resuspended in 20  $\mu\text{l}$  of Tris pH 7.5 and 2  $\mu\text{l}$  aliquots reverse transcribed in a 20  $\mu\text{l}$ -reaction mixture containing 150 ng of random hexanucleotides, 2 mM dNTPs, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM dithiothreitol and 200 U SuperScript II RNase H<sup>–</sup> reverse transcriptase (Life Technologies, Paisley, U.K.). PCR experiments were performed with 2  $\mu\text{l}$  of the first-strand DNA in a 25  $\mu\text{l}$  reaction mixture containing 200  $\mu\text{M}$  dNTPs, 1.5 mM MgCl<sub>2</sub>, 25 pmol of specific forward and reverse primers and 1 U of recombinant Taq DNA polymerase (Life Technologies, Paisley, U.K.) and amplified for 30 cycles (Biometra-TRIO thermocycler): 95°C for 1 min, 60°C for 2 min and 72°C for 2 min followed by a final extension step at 72°C for 10 min. Rat B<sub>1</sub> receptor cDNA was amplified with the sense primer (5'-GCGGAAATCTACCTGGCTAACTTG-3') at base position 392–415 and antisense primer (5'-CAGTCACGGGGAG-GAGGAAACC-3') at base position 806–827 of rat B<sub>1</sub> receptor cDNA (Ni *et al.*, 1998). The quality of RNA samples was evaluated by RT-PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers (sense primer, 5'-AAGGTGAAGGTCGGAGTCAACG-3' (position 8–29); antisense primer 5'-GGCAGAGATGATGACCCTTTT-GGC-3' (position 362–339). A plasmid containing the mouse B<sub>1</sub> receptor cDNA (Pesquero *et al.*, 1996) was used as the template for the positive PCR control reaction. PCR products were detected by electrophoresis on 2% agarose gels stained with ethidium bromide. The sequence of the amplified cDNA was verified using direct automated DNA sequencing using the TaqDyeDeoxy terminator cycle sequencing method (Thermo-sequence, Amersham Ltd, U.K.).

#### Reagents

All reagents were purchased from Sigma Chemical Co. (Dorset, U.K.), unless otherwise indicated below. Lipopolysaccharide (*E. coli* 055:B5) was purchased from Becton Dickinson (Oxford, U.K.). Des-Arg<sup>10</sup>HOE140 was purchased from RBI (Dorset, U.K.). HOE140 was a kind gift from Hoechst (Frankfurt, Germany) and rat recombinant IL-1 $\beta$  a generous gift from Dr R.C. Newton (Du Pont-Merck, Wilmington, DE, U.S.A.). ODQ was synthesised in The Wolfson Institute for Biomedical Research (London, U.K.).

#### Data analysis

The responses to DABK and BK are expressed as a percentage of their individual maxima. ED<sub>50</sub> values were calculated from the 50% response level and expressed as geometric mean with 95% confidence limits in parentheses using a non-linear curve fitting analysis. The potency of the antagonists was expressed as a pA<sub>2</sub> value. Apparent pA<sub>2</sub> values were calculated using

single point analysis from  $\text{pA}_2 = \log(\text{DR}-1) - \log[\text{B}]$ , where DR is the dose-ratio of agonist measured in the absence and presence of antagonist (B). The assumption of simple competition (i.e. slope of unity) between antagonist and agonist was checked with the Schild plot. All other data are given as arithmetic means  $\pm$  s.e.mean or geometric mean with 95% confidence limits. The number of observations is indicated by *n*. The significance of differences between the values was determined by use of Student's paired or unpaired *t*-test. Dose-response curves were compared using two-way ANOVA (analysis of variance, Graph Pad Prism, Graph Pad Software Inc., San Diego, U.S.A.). The criterion for statistical significance was set at  $P < 0.05$ .

## Results

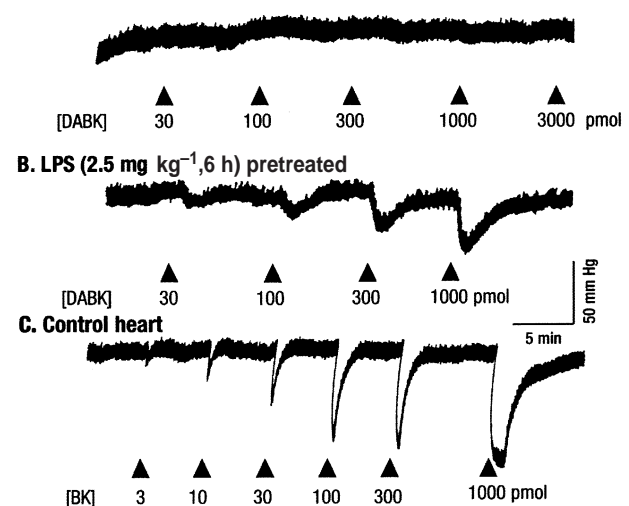
#### Effects of endotoxin treatment

Treatment of rats with LPS produced characteristic symptoms: pilo-erection, malaise and loose stools. Hearts from LPS-treated rats displayed significantly ( $P < 0.05$ ) higher basal CPP values than control hearts (CPP (mm Hg) =  $66 \pm 3$  (control,  $n = 42$ );  $114 \pm 5$  ( $P < 0.05$ , 6 h,  $n = 40$ );  $111 \pm 14$  (14 h,  $n = 4$ );  $94 \pm 8$  ( $P < 0.05$ , 24 h,  $n = 5$ ).

#### Influence of endotoxin treatment on coronary reactivity to DABK and BK

The B<sub>1</sub> receptor selective agonist DABK had no effect on CPP in hearts from control rats ( $n = 6$ , Figures 1 and 2). Elevation of CPP with endothelin-1 (CPP =  $112.7 \pm 15.2$ ,  $n = 4$ ) failed to reveal a response to DABK. In contrast, BK produced dose-dependent vasodilations (Figure 1, ED<sub>50</sub> = 37.9 pmol (4.4–329.1); E<sub>max</sub> = 55.4 mm Hg (40.1–70.7),  $n = 13$ ) in control hearts with endothelin-1-elevated CPP. The B<sub>2</sub> receptor antagonist, HOE140 had no effect on basal CPP *per se* but caused significant rightward shifts ( $P < 0.05$ , 2-way ANOVA) of the dose-response curve to BK in a competitive fashion without affecting the maximum response (pA<sub>2</sub> =  $10.1 \pm 1.5$ ,

#### A. Control heart



**Figure 1** Typical traces showing vasodilator effects of bradykinin (BK) and des-Arg<sup>9</sup>BK (DABK), measured as decreases in coronary perfusion pressure in the rat isolated heart. (A and C) control heart; (B) heart from LPS (2.5 mg kg<sup>–1</sup>, 6 h)-pretreated rat.

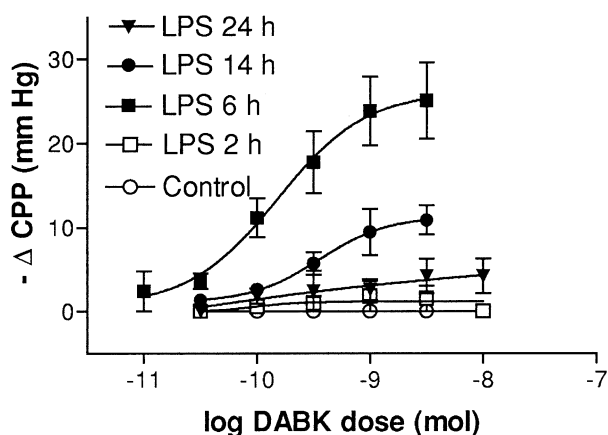
Schild slope =  $0.96 \pm 0.2$ ,  $n = 13$ ); the B<sub>1</sub> receptor antagonist, DALBK (100 nM) had no significant effect on the dose-response curve to BK ( $P > 0.05$ , 2-way ANOVA,  $n = 4$ ).

Pretreatment of rats with LPS promoted a time-dependent induction of coronary vasodilation responses to DABK. Whilst DABK had no effect on CPP in hearts taken from rats 2 h post LPS ( $n = 4$ ), marked dose-dependent reductions in CPP were observed after 6 h LPS treatment ( $ED_{50} = 149.4$  pmol ( $33.0 - 675.6$ );  $E_{max} = 27.0$  mm Hg ( $15.7 - 36.6$ ),  $n = 9$ ). At the 14 and 24 h time points the  $E_{max}$  of the dose-response curves to DABK were significantly reduced ( $P > 0.05$ , two-way ANOVA) relative to the 6 h time point; values were reduced by approximately 50 and 80% respectively (Figure 2). DALBK (30 nM) had no effect on basal CPP (6 h post-LPS) but caused a significant ( $P < 0.05$ , two-way ANOVA) parallel rightward shift of the dose-response curve to DABK (apparent  $pA_2 = 8.4 \pm 0.16$ ,  $n = 5$ , Figure 3) without affecting the maximum response. In contrast HOE140 (10 nM) did not affect either basal CPP or the dose-response curve to DABK ( $P > 0.05$ , two-way ANOVA,  $n = 4$ , Figure 3).

$E_{max}$  values of dose-response curves to BK, in hearts from rats 6 h post LPS ( $ED_{50} = 76.0$  pmol ( $27.2 - 212.2$ ),  $E_{max} = 49.6$  mm Hg ( $39.1 - 60.0$ ),  $n = 5$ ) appeared to be reduced relative to controls, i.e. control hearts with endothelin-1-elevated CPP, but this effect did not reach statistical significance ( $P > 0.05$ , two-way ANOVA). The BK dose-response curves in hearts from rats 6 h post LPS were unaltered ( $P > 0.05$ , two-way ANOVA) by DALBK (30 nM) but were significantly inhibited ( $P < 0.05$ , two-way ANOVA) by HOE140 (10 nM) without reducing the maximum response (apparent  $pA_2 = 9.5 \pm 0.7$ ,  $n = 5$ ).

#### Mechanisms of kinin responses

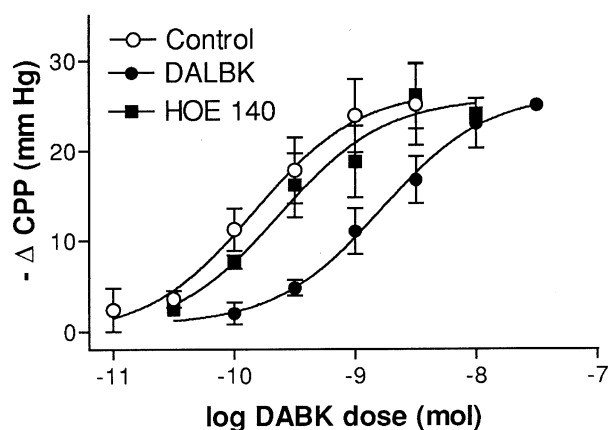
**Endothelium removal** Endothelium removal produced an increase in CPP in hearts from both untreated rats and rats 6 h post-LPS treatment of  $33.7 \pm 6.1$  mm Hg ( $n = 5$ ) and  $23.3 \pm 4.2$  mm Hg ( $n = 5$ ) respectively ( $P > 0.05$ ). Figure 4 shows that removal of the endothelium caused a significant ( $P < 0.05$ ) reduction in responses to DABK (1 nmol) and acetylcholine (3 nmol,  $P < 0.05$ ) but had no effect on the responses to SNP (10 nmol,  $P > 0.05$ ,  $n = 5$ ).



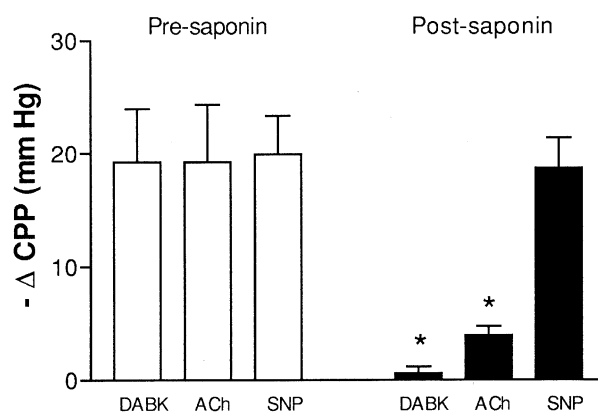
**Figure 2** Time-dependent induction of coronary vasodilation responses to des-Arg<sup>9</sup>BK (DABK), measured as decreases in coronary perfusion pressure ( $\Delta$ CPP). Hearts were obtained from control or LPS ( $2.5$  mg  $kg^{-1}$ )-pretreated rats at 2, 6, 14 and 24 h post treatment. Data are means  $\pm$  s.e.mean ( $n = 4 - 9$ ).

**Involvement of endothelial mediators** ODQ, whilst producing an increase in CPP of  $12.8 \pm 3.7$  mm Hg ( $n = 5$ ), had no significant effect on the DABK-induced dose-response curves ( $P > 0.05$ , two-way ANOVA, Figure 5). ODQ did however completely abolish the dilator response to SNP (10 nmol,  $n = 4$ , control response =  $-20 \pm 4.2$  mm Hg; no detectable response following ODQ treatment). L-NAME ( $n = 5$ ) also increased CPP ( $34.3 \pm 4.3$  mm Hg) and reduced the response to acetylcholine (3 nmol) by  $75 \pm 6.2\%$  ( $n = 4$ ), but did not modify the dose-response curve to DABK ( $P > 0.05$ , two-way ANOVA, Figure 5).

Indomethacin completely abolished the responses to submaximal doses of DABK ( $< 1$  nmol) and inhibited the maximal response to DABK by 85% (see Figure 5). Additionally, DABK caused a dose-dependent increase in the concentration of 6-keto-PGF<sub>1 $\alpha$</sub>  measured in the perfusate ( $E_{max} = 0.3 \pm 0.04$  ng  $ml^{-1}$ ) of hearts from rats 6 h post-LPS



**Figure 3** Comparison of the effects of kinin B<sub>1</sub> and B<sub>2</sub> receptor antagonism on the vasodilator responses to des-Arg<sup>9</sup>BK (DABK). Vasodilator responses are expressed as decreases in coronary perfusion pressure ( $\Delta$ CPP) in hearts from LPS ( $2.5$  mg  $kg^{-1}$ , 6 h)-pretreated rats. Data obtained in the absence or presence of des-Arg<sup>9</sup>-[Leu<sup>8</sup>]-BK (DALBK, B<sub>1</sub> receptor antagonist, 30 nM) and HOE140 (B<sub>2</sub> receptor antagonist, 10 nM). Each point represents the means  $\pm$  s.e.mean ( $n = 4 - 9$ ).

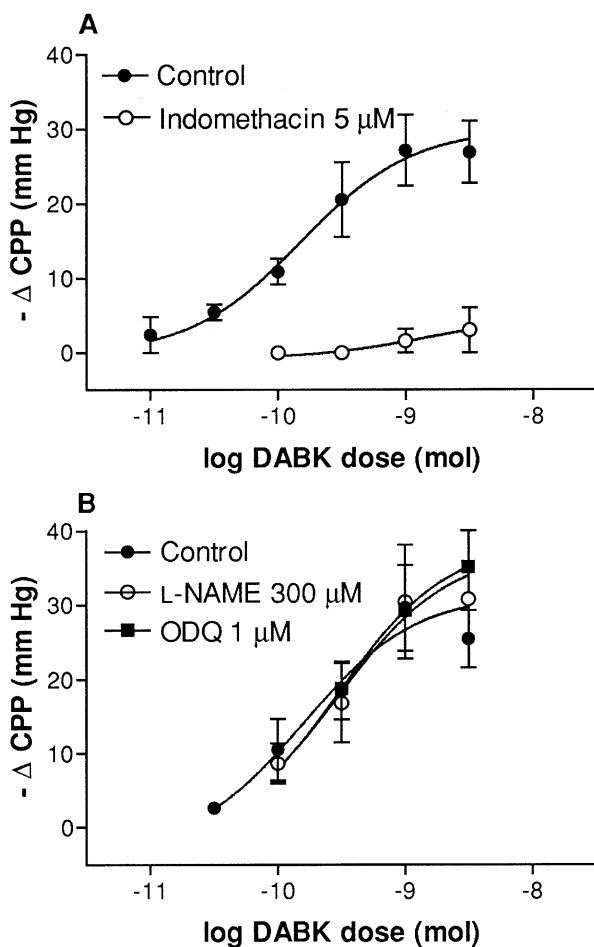


**Figure 4** Effects of endothelium removal on des-Arg<sup>9</sup>bradykinin (DABK)-induced coronary vasodilation. Vasodilator responses are expressed as changes in coronary perfusion pressure ( $\Delta$ CPP), mm Hg in response to DABK (1 nmol), acetylcholine (ACh, 3 nmol) and sodium nitroprusside (SNP, 10 nmol) before (open columns) and after (solid columns) saponin treatment in hearts from LPS ( $2.5$  mg  $kg^{-1}$ , 6 h)-pretreated rats. Data are means  $\pm$  s.e.mean ( $n = 5$ ). \* $P < 0.05$  vs pre-saponin values.

(Figure 6). No increase in 6-keto-PGF<sub>1 $\alpha$</sub>  levels were observed following DABK administration in hearts from untreated rats ( $n=3$ , all doses  $P<0.06$  ng ml<sup>-1</sup>).

#### Mechanisms of B<sub>1</sub> receptor induction

*In vitro* perfusion of control hearts with LPS ( $n=6$ ) produced no effect *per se* on CPP. Perfusion with the inflammatory cytokine, IL-1 $\beta$  ( $n=6$ ) caused a gradual, transient (30 min) reduction in CPP of  $33.8\pm 4.0$  mm Hg ( $n=6$ ). Figure 7 shows that both treatments induced a time-dependent induction of a vasodilator response to DABK (1 nmol); the responses peaking at 3 h (IL-1 $\beta$ ) and >4 h (LPS). These responses to DABK were completely abolished by indomethacin treatment ( $n=5$ ) and inhibited by 68.5 $\pm$ 7.6% (LPS,  $n=4$ ) and 67.2 $\pm$ 3.9% (IL-1 $\beta$ ,  $n=5$ ) in the presence of DALBK (30 nM). No response to DABK was observed in control hearts perfused for 4 h in the absence of LPS or IL-1 $\beta$ . The initial vasodilator response to BK (0.1 nmol,  $54.3\pm 4.4$  mm Hg,  $n=6$ ) was not significantly ( $P>0.05$ ) affected 3 h after the IL-1 $\beta$  perfusion however a 27% reduction was observed after 4 h of LPS treatment ( $P<0.05$ ). The SNP (10 nmol)-induced vasodilation (control response =  $25\pm 4.9$  mm Hg,  $n=4$ ) was not significantly affected ( $P>0.05$ ) by either treatment.



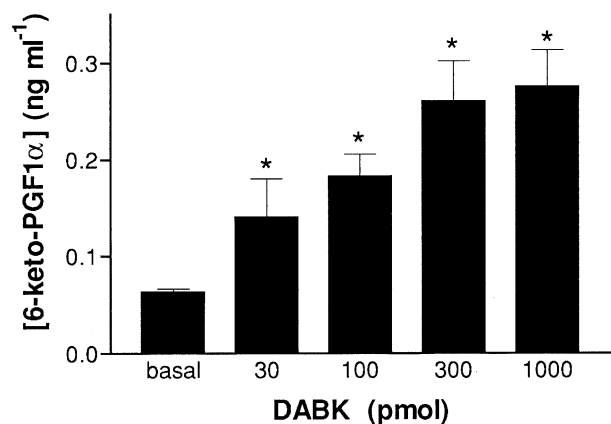
**Figure 5** Effects of inhibition of (A) cyclooxygenase and (B) the nitric oxide-guanylyl cyclase pathway on des-Arg<sup>9</sup>bradykinin (DABK)-induced coronary vasodilation. Vasodilator responses are expressed as changes in coronary perfusion pressure ( $\Delta$ CPP, mmHg) produced by DABK in hearts from LPS ( $2.5$  mg kg<sup>-1</sup>, 6 h)-pretreated rats. Data obtained in the absence or presence of (A) indomethacin ( $5$   $\mu$ M), (B) L-NAME ( $300$   $\mu$ M) or ODQ ( $1$   $\mu$ M). Each point represents the means  $\pm$  s.e.mean ( $n=5-7$ ).

#### Molecular expression of B<sub>1</sub> receptors

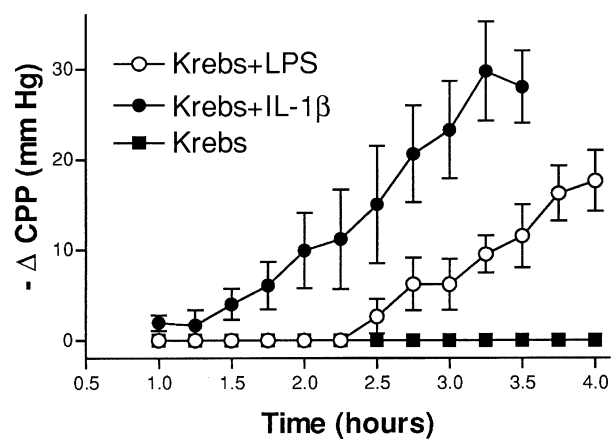
RT-PCR of cardiac mRNA from untreated rats or rats 1 h post-LPS treatment revealed no expression of B<sub>1</sub> receptor mRNA. In contrast, B<sub>1</sub> receptor mRNA was consistently detected in hearts from rats 3 and 6 h post LPS (Figure 8A).

B<sub>1</sub> receptor mRNA was also detected in hearts perfused *in vitro* with LPS or IL-1 $\beta$  whereas no expression was detected in non-perfused control hearts (Figure 8B). Hearts perfused for 4 h in the absence of LPS or IL-1 $\beta$  also expressed B<sub>1</sub> receptor mRNA. GAPDH mRNA was detected in all hearts tested.

The resulting PCR products derived from two separate samples using B<sub>1</sub> receptor specific primers were subjected to automated sequencing. The nucleotide sequence of the amplified cDNA revealed identity with the rat B<sub>1</sub> receptor cDNA sequence (Ni *et al.*, 1998).



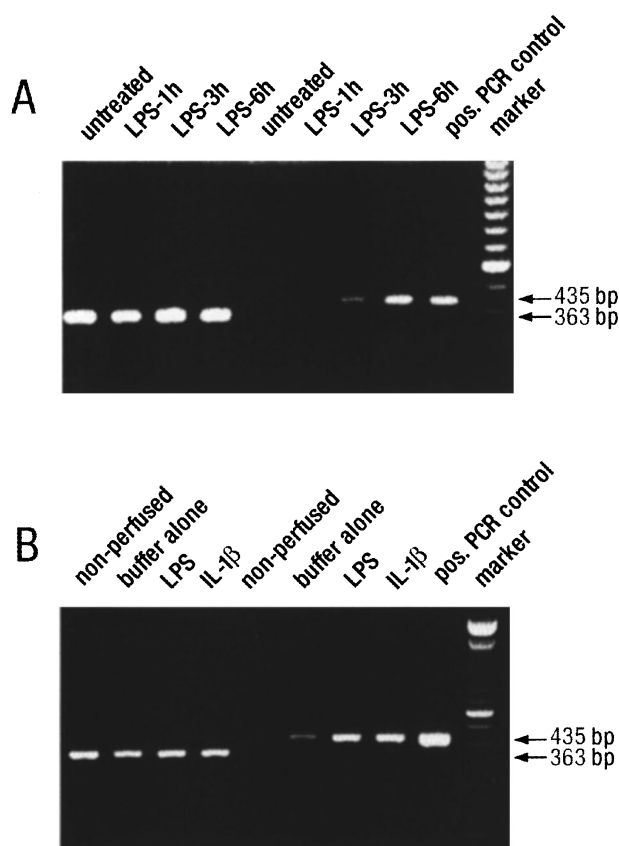
**Figure 6** Increases in coronary perfusate concentrations of 6-keto-PGF<sub>1 $\alpha$</sub>  in response to bolus doses of des-Arg<sup>9</sup>BK (DABK) in hearts from LPS ( $2.5$  mg kg<sup>-1</sup>, 6 h)-pretreated ( $n=6$ ) rats. Data shown are the means  $\pm$  s.e.mean of 6-keto-PGF<sub>1 $\alpha$</sub>  concentrations in perfusate collected 2 min before and 2 min after administration of des-Arg<sup>9</sup>BK. \* $P<0.05$  vs control.



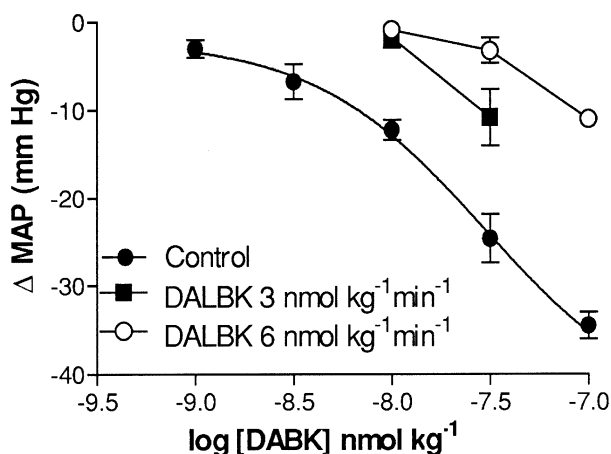
**Figure 7** *In vitro* perfusion of LPS or IL-1 $\beta$  sensitises rat hearts to des-Arg<sup>9</sup>bradykinin (DABK). Control rat hearts were perfused with Krebs ( $n=4$ ) or Krebs plus LPS ( $1$   $\mu$ g ml<sup>-1</sup>, 4 h,  $n=6$ ) or IL-1 $\beta$  ( $5$  ng ml<sup>-1</sup>, 2 h,  $n=6$ ); vasodilator responses to repeated doses of DABK ( $1$  nmol) were measured as decreases in coronary perfusion pressure (CPP). Data points represent means  $\pm$  s.e.mean.

### Effects of kinins on blood pressure in vivo

MAP of control untreated rats was  $125.9 \pm 2.5$  mm Hg ( $n = 8$ ). To compare the activity of DABK in control vs LPS-treated rats MAP in LPS-treated rats was raised using noradrenaline.



**Figure 8** B<sub>1</sub> receptor (435 bp) and GAPDH (363 bp) PCR products amplified from poly(A)<sup>+</sup> RNA extracted from (A) non-perfused hearts from either untreated or LPS (2.5 mg kg<sup>-1</sup>, 1, 3 or 6 h)-treated rats and (B) hearts from untreated rats perfused *in vitro* with either LPS (1 μg ml<sup>-1</sup>, 4 h), IL-1β (5 ng ml<sup>-1</sup>, 2 h) or buffer solution alone (4 h). Positive PCR control is a mouse B<sub>1</sub> receptor cDNA plasmid.



**Figure 9** The hypotensive response ( $\Delta$ MAP) to des-Arg<sup>9</sup>bradykinin (DABK) and its antagonism by des-Arg<sup>9</sup>[Leu<sup>8</sup>]-BK in LPS (2.5 mg kg<sup>-1</sup>, 6 h)-pretreated rats. Data obtained in the absence or presence of des-Arg<sup>9</sup>[Leu<sup>8</sup>]-BK (3, 6 nmol kg<sup>-1</sup> min<sup>-1</sup>). Each point represents the means  $\pm$  s.e.mean ( $n = 4-7$ ).

MAP of LPS (2.5 mg kg<sup>-1</sup>, 6 h)-treated rats infused with noradrenaline was  $103.9 \pm 3.3$  mm Hg ( $n = 10$ ,  $P > 0.05$  relative to controls). Bolus doses of DABK (1–100 nmol kg<sup>-1</sup>) had no effect on MAP in control untreated rats ( $n = 4$ ) but caused dose-dependent decreases in MAP in LPS (2.5 mg kg<sup>-1</sup>, 6 h)-treated rats with an ED<sub>50</sub> of 29.7 (8.3–105) nmol kg<sup>-1</sup> and E<sub>max</sub> of 45.1 (24–66) mm Hg ( $n = 10$ , Figure 9). DALBK (3, 6 nmol kg<sup>-1</sup> min<sup>-1</sup>, 5 min) significantly ( $P < 0.05$ ) reduced the hypotensive effect of DABK (Figure 9). DALBK (3 nmol kg<sup>-1</sup> min<sup>-1</sup>, 5 min) produced no effect *per se* in control animals but in LPS (10 mg kg<sup>-1</sup>, 6 h)-treated rats it increased MAP by  $5.3 \pm 1.2\%$  ( $n = 6$ ,  $P < 0.05$ ). The B<sub>1</sub> receptor antagonist des-Arg<sup>10</sup>HOE 140 (30 nmol kg<sup>-1</sup> min<sup>-1</sup>, 5 min) also increased MAP by  $8.8 \pm 0.34\%$  ( $n = 5$ ,  $P < 0.05$ ) in LPS (10 mg kg<sup>-1</sup>, 6 h)-treated rats. Captopril (1 mg kg<sup>-1</sup>) reduced MAP by  $11.1 \pm 1.9\%$  ( $n = 4$ ,  $P < 0.05$ ) in LPS (10 mg kg<sup>-1</sup>, 6 h)-treated rats. This effect was reversed by des-Arg<sup>10</sup>HOE 140 (30 nmol kg<sup>-1</sup> min<sup>-1</sup>) treatment ( $P < 0.05$ ).

### Discussion

The present study has shown that the kinin B<sub>1</sub> receptor, whilst not basally expressed, is induced by endotoxin in the rat coronary circulation. This was demonstrated both in terms of mRNA expression as detected by RT-PCR and of a coronary vasodilation response to DABK. Functional responses to DABK increased over time, in parallel with the mRNA expression, peaking at 6 h and almost absent by 24 h post-endotoxin treatment. The coronary vasodilation responses to DABK were B<sub>1</sub> receptor-mediated and endothelium-dependent. Furthermore the responses were shown to be mediated by prostanoids but did not involve NO. *In vitro* treatment with endotoxin revealed that the receptor is induced in the coronary circulation in the absence of circulating cells, possibly by a direct effect of endotoxin within the blood vessel wall.

#### Induction of B<sub>1</sub> receptor-mediated responses and functional relevance in vivo

A single bolus exposure to endotoxin resulted in the development of a functional response to DABK that was maximal at 6 h and almost absent by 24 h. The loss of response to DABK at 24 h may reflect the differences between a continued inflammatory stimulus as opposed to single exposure as in the present study. Whether the functional response is sustained in the presence of a prolonged inflammatory stimulus is unclear and warrants further investigation. The B<sub>2</sub> receptor-mediated response to BK was not significantly affected 6 h following endotoxin treatment suggesting that B<sub>2</sub> receptor regulation does not accompany B<sub>1</sub> receptor induction at this time point in this model. However further studies are required to confirm this.

Whilst it is clear that NO plays a major role in the hypotension associated with endotoxemia there is also a significant NO-independent contribution to this response (Ungureanu-Longrois *et al.*, 1995). Our studies suggest that activation of endotoxin-induced B<sub>1</sub> receptors may have a role to play in mediation of the latter component. Indeed the time course of iNOS induction in endotoxemia in the rat is similar to that found for the B<sub>1</sub> receptor in the present study (Luss *et al.*, 1995; Kinugawa *et al.*, 1997). This supports our initial hypothesis that the *de novo* induction of coronary B<sub>1</sub> receptors contributes to an NO-independent regulation of coronary tone and this regulation may have relevance *in vivo*.

Support for the hypothesis that the induction of the B<sub>1</sub> receptor occurred at the level of a specific receptor-mediated response was provided by B<sub>1</sub> receptor selective agonist and antagonist studies. The B<sub>1</sub> receptor selective antagonist DALBK (Marceau *et al.*, 1998) inhibited the DABK-induced coronary vasodilations with an apparent pA<sub>2</sub> value of 8.4 and was active against the DABK-induced hypotension *in vivo*. A partial agonist action of DALBK was also observed both in the isolated heart and on blood pressure which is consistent with its activity at rat B<sub>1</sub> receptors (Marceau *et al.*, 1998). The B<sub>2</sub> receptor selective antagonist, HOE140, whilst effectively inhibiting the BK-induced coronary vasodilations in both control and endotoxemic hearts did not affect the response to DABK. Whilst these results strongly suggest a specific B<sub>1</sub> receptor-mediated response further studies are required to fully confirm induction at the level of receptor protein.

Basal coronary perfusion pressure was elevated in hearts from endotoxin treated rats compared to controls. It has been suggested that this may be due to release of endothelin-1 (Hohlfeld *et al.*, 1995). The possibility that elevation of perfusion pressure alone was responsible for the development of responses to DABK was excluded, since raising coronary perfusion pressure with endothelin-1 in control hearts did not reveal a response to DABK whilst responses to BK remained unchanged. This was further demonstrated in hearts from rats 2 h post-endotoxin in which coronary perfusion pressure was elevated relative to controls yet no response to DABK was observed.

#### *Mechanisms of B<sub>1</sub> receptor-mediated coronary vasodilation*

DABK-induced vasodilation was endothelium-dependent since removal of the endothelium with saponin abolished the response. This loss of response was not due to damage of the smooth muscle since responses to the endothelium-independent vasodilator, SNP, remained unchanged. These results suggest that the induced B<sub>1</sub> receptor expression, in this particular situation, is probably localized to the endothelium. However confirmation of this will require further studies. These results are in keeping with reports showing endothelium-dependency of induced B<sub>1</sub> receptor-mediated responses in various other blood vessels (Drummond & Cocks, 1995b; Deblois & Marceau, 1987) including human isolated coronary arteries (Drummond & Cocks, 1995a).

NO did not play a significant role in mediation of these responses since inhibition of NO synthase had no effect on DABK-induced vasodilation. This is in agreement with the study by Nicolau *et al.* (1996) who show that the DABK-induced hypotension in endotoxin-treated rats was not affected by NO synthase inhibition. However, it is possible that stimulated NO release from preformed stores could be involved in this response. We found that the guanylyl cyclase inhibitor ODQ, at concentrations shown to inhibit NO-mediated effects (this study; Muller *et al.*, 1998), was also inactive suggesting that the activation of guanylyl cyclase downstream is also not involved. Moreover the observation that the cyclooxygenase inhibitor, indomethacin, abolished the response to DABK suggests that prostanoids, most likely PGI<sub>2</sub>, are the primary if not sole mediator of the response. In support of this, we observed a dose-dependent elevation of 6-keto-PGF<sub>1 $\alpha$</sub>  levels in the effluent following DABK administration. The involvement of endothelial PGI<sub>2</sub> in DABK-induced dilator responses is in agreement with data obtained in other isolated vascular beds; including rabbit mesenteric (Churchill & Ward, 1986) and dog renal (Rhaleb *et al.*, 1989) arteries. However, there are also conflicting studies excluding prosta-

noid involvement in DABK-induced vasorelaxation (Pruneau & Belichard, 1993; Pruneau *et al.*, 1996; Santiago *et al.*, 1995; DeWitt *et al.*, 1994) and hypotension (Nicolau *et al.*, 1996). This difference may be related to species and regional variation or to the absence of specific prostanoid receptor subtypes.

#### *Mechanisms of B<sub>1</sub> receptor induction*

*In vitro* LPS perfusion of normal hearts caused a time-dependent induction of functional reactivity to DABK, associated with B<sub>1</sub> receptor mRNA expression, in a similar manner to that achieved with *in vivo* LPS treatment. These effects appear to be selective for LPS treatment as DABK was inactive in hearts not perfused with LPS. Interestingly, unlike the *in vivo* situation LPS did not raise CPP *in vitro* suggesting that this is an *in vivo* phenomenon. However this requires further investigation. These results suggest that induction of B<sub>1</sub> receptors in the coronary vasculature *in vivo* is most likely independent of circulating cells and is probably due to a direct effect of LPS on the blood vessel. Indeed experimental neutropenia has no effect on induction of rabbit aortic B<sub>1</sub> receptors by *in vivo* endotoxin treatment (Bouthillier *et al.*, 1987).

Inflammatory cytokines including IL-1 $\beta$  have also been implicated in the pathogenesis of endotoxemia (Bellomo, 1992; Waage *et al.*, 1991; Martich *et al.*, 1993) and are the endogenous trigger for the induction of kinin B<sub>1</sub> receptors in various systems (Marceau *et al.*, 1998). The present study supports this proposal since following perfusion of hearts with IL-1 $\beta$  vasodilator responses to DABK were observed in parallel with B<sub>1</sub> receptor expression. Since *in vitro* IL-1 $\beta$  perfusion caused B<sub>1</sub> receptor induction earlier than that achieved by LPS perfusion it is possible that local production of this cytokine, a key mediator in several LPS-induced responses (Glauser, 1996) mediates the *in vivo* and *in vitro* effects of LPS, however this requires further investigation for confirmation. Demonstration of B<sub>1</sub> receptor mRNA expression in control hearts perfused for 4 h, in the absence of added endotoxin, indicates that the experimental process is sufficient stimulus to cause spontaneous B<sub>1</sub> receptor induction as found in other *in vitro* systems (Deblois & Marceau, 1987; Drummond & Cocks, 1995a,b). However, this modest expression of mRNA was not linked to a functional response to DABK at this time point.

#### *Conclusion*

We have shown that endotoxin treatment induces *de novo* functional B<sub>1</sub> receptor expression in the rat coronary circulation possibly *via* a direct action within the vessel wall independent of circulating cells. The B<sub>1</sub> receptor-mediated coronary vasodilation is dependent on an intact endothelium and occurs as a result of the secondary release of prostanoids and is independent of NO. The current findings are entirely consistent with the hypothesis that *de novo* B<sub>1</sub> receptor induction plays a role in the regulation of the coronary circulation during endotoxemia and also suggests that B<sub>1</sub> receptor induction may contribute to the hypotension associated with this pathological condition.

This study and Dr P.G. McLean were supported by the British Heart Foundation (BHF, PG#97013). Dr Ahluwalia is funded by a BHF Intermediate Fellowship and Dr Perretti is a Research Fellow of the Arthritis Research Campaign. The authors kindly thank Dr J.B. Pesquero (Escola Paulista de Medicina, Sao Paulo, Brazil) for the supply of the B<sub>1</sub> receptor plasmid.

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(Received March 19, 1999  
Revised April 16, 1999  
Accepted June 1, 1999)