BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **243,** 246–252 (1998)
ARTICLE NO. RC977999

B1 Bradykinin Receptors and Carboxypeptidase M Are Both Upregulated in the Aorta of Pigs after LPS Infusion

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Received December 12, 1997

Bradykinin receptor subtypes were characterized in aortic cryosections obtained from healthy normal pigs, animals that were given an LPS infusion, and animals that came with a pre-existing infection or inflammation to the laboratory by binding studies and *in vitro* autoradiography. In control aorta a single class of high affinity B2 binding sites, located within the endothelium, but with no significant binding of B1 ligand were identified. No major changes in the expression of B2 BK receptors were noted in inflammed tissues. In cryosections of inflammed vascular tissue a markedly increased endothelial carboxypeptidase M activity was verified that parallelled an upregulation of B1 receptors in the aortic smooth muscle layer. In crosstalk between endothelial cells and smooth muscle cells B1 receptor mediated functional responses may counteract some of the detrimental effects of inflammation.

Kinins are vasoactive peptides which participate in a wide range of vascular functions through their action via B2 and B1 BK receptors. B2 BK receptors are known to be constitutively expressed in a wide range of tissues including endothelial cells from arteries of different species [1,2]. However, the amount and extent of possible variation of vascular endothelial B2 receptor number during inflammatory conditions has not been studied so far. BK B1 receptors, with few exceptions not being present under normal circumstances, are reported to be upregulated or newly synthesized both *in vivo* and *in vitro* by a wide variety of noxious stimuli, cytokines (interleukin-1) or endotoxins such as LPS from *Salmonella abortus equi* [3,4,5]. In particular this phenomenon has been demonstrated in various preparations of blood vessels that have been used to define this receptor subtype by classical pharmacological studies [6]. Bacterial LPS has been used in a porcine model to experimentally induce bacteremia and sepsis [3]. This was obviously resulting in the *de novo* appearance of B1 BK receptors as evidenced by a markedly decreased blood pressure response after an intraarterial bolus injection of des-Arg9-BK [7]. Interestingly, the same effect was also noted in pigs suffering from inflammatory conditions due to naturally occurring disease. In this model therapeutic application of B2 receptor antagonists, but not B1 antagonists seem to be beneficial in terms of outcome [8,9]. The functional impact of B1 receptor upregulation on blood vessel regulation during LPS-shock and the reasons for the differing effects of B2 and B1 receptor antagonists are as yet poorly explained. Therefore we utilized vascular tissues derived from this model to address the question, which cell types within the vessel wall are involved in these phenomena and to clarify the extent of B1 BK receptor upregulation during the different experimental conditions. For this purposes we made use of the method of receptor autoradiography, which localizes binding sites *in situ* in structurally intact tissues and provides an excellent means of targeting BK action [10,11,12,13]. A potential function of B1 BK receptors in inflammatory processes or processes following LPS-treatment also requires the physiological provision of appropriate amounts of specific B1 receptor ligands des-Arg9-BK and des-Arg10-kallidin that are physiologically formed in blood and in tissues as a result of enzymatic cleavage of BK or

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Abbreviations: LPS, lipopolysaccharide; BK, bradykinin.

0006-291X/98 $25.00

kallidin by carboxypeptidase enzymes [5,14,15]. Therefore the up- or downregulation of carboxypeptidase activity may modify kinin action on vessel cells under different pathological conditions such as inflammation [15].

In this study we show that B1 BK receptors and carboxypeptidase M activity are both upregulated - although at different cellular sites - as a consequence of either pre-existing infection or LPS-induced inflammation.

MATERIALS AND METHODS

Reagents. Radio-labelled BK ([2,3-prolyl-3,4-^{3}H(*N*)]-BK), (78.4 and 121.6 Ci/mmol) and des-Arg10-kallidin ([des-Arg10], [3,4-Prolyl-3,4-^{3}H(*N*)]-kallidin), (110 Ci/mmol) were from New England Nuclear. *Mergetpa* (DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic Acid) was from Calbiochem (Bad Soden, Germany), and Hoe 140 as a kindly gift from Dr. Schoelkens (Hoechst, Frankfurt, Germany). Unlabelled agonists and antagonists, as well as protease inhibitors and all other chemicals were obtained from Sigma Chemie (Deisenhofen, Germany). Photosensitive emulsion for high resolution microautoradiography (LM 1 for light microscopy) was from Amersham Buchler.

Animal model and preparation of tissues. A pig model for functional studies of the kinin-kallikrein-system in experimentally LPS-induced inflammation was used as described previously [7]. Aortic vessels were removed either from healthy pigs or pigs with LPS shock, that have been pre-treated with endotoxin from *Salmonella abortus equi* (2μg/kg/h i.v.) for 4h. Thoracic aorta was also prepared from animals that had been routinely checked for signs of pre-existing disease before the experiment and were classified as sick (pre-existing disease) if any of the following signs were found: Abscess or any other major skin infection, râles on auscultation, pulmonary artery mean pressure greater than 25mm Hg, peak airway pressure less than 25mbar, body temperature greater than 38°C, haemoglobin less than 7.0g/100ml, and white blood cell count less than 5,000 or greater than 18,000/μl. Healthy control animals receiving NaCl infusion instead of LPS infusion served as control.

Removed tissue samples were rinsed several times in saline and immediately frozen in liquid nitrogen. 10μm or 20μm cryostat sections were cut at −30°C and thaw-mounted on gelatine-coated slides. Sections were dried overnight in a desiccator at 4°C and either processed for immediate use or stored at −80°C. Protein content of cryosections was determined using a commercial protein microassay (Bio-Rad, Munich, Germany).

Receptor binding procedure. Cryosections were incubated for 2h in an icebath (4°C) in 25mM TES, pH 6.8, sucrose 300mM, BSA 2%, DTT 1mM, supplemented with a protease inhibitor mixture consisting of bacitracin 2mM, 1.10-phenanthroline 1μM, captopril 10μM, phosphoramidon 10μM. Binding studies were carried out by additon of nearly saturating concentrations of [^{3}H]BK or [^{3}H]des-Arg10-kallidin as B2 and B1 BK receptor agonists respectively in the presence (non-specific binding) or absence (total binding) of an excess (1μM) of unlabelled agonists. Incubations were terminated by two cycles of rapid washing of cryosections in washing buffer (25mM TES buffer including 0.2% bovine serum albumin and the inhibitors described above) in order to remove excess of free radioligand and were finally briefly dipped into distilled water to remove salts.

For quantitation of receptor bound [^{3}H]ligand sections were wiped off with filterpaper and transferred to liquid scintillation vials. The extent of BK degradation on cryostat sections under various conditions was assessed according to Roscher et al. [16].

Receptor autoradiography. High resolution receptor microautoradiography was carried out as described previously [12]. For visualization of ligand binding on cryostat section tissue sections were dried and fixed with paraformaldehyde, defatted and exposed to emulsion coated coverslips for approximately 8 weeks.

Carboxypeptidase M assay. Carboxypeptidase M activity was evaluated according to the method of Tan et al. [17].

The assay is based on the fluorescent substrate dansyl-Ala-Arg and utilizes the ability to extract the product, dansyl-Ala, into chloroform while the remaining substrate stays in the aqueous phase at acidic pH. The fluorescence is measured at 340nm excitation and 495nm emission wavelength.

Four 20μm cryosections (in the presence of endothelium or devoid of it) were placed into an *Eppendorf* tube containing 125μl of buffer, 0 or 25μl of 100μM *Mergetpa*, and 75 or 50μl water to give a final volume of 200μl and kept on ice. After homogenizing three strokes for 3 seconds each with a Branson B-10 sonifier (Branson Power Company, Danbury, Connecticut, USA) the homogenate was preincubated for 10min on ice. Then 50μl of 1.0mM dansyl-Ala-Arg substrate (a kindly gift from Dr. Skidgel, University of Illinois at Chicago) were added and mixed to start the reaction. Samples were incubated at 37°C for 15min to 3h and the reaction was stopped thereafter by adding 150μl 1.0M citrate, pH 3.1. At least 1ml chloroform was added to each tube, mixed vigorously for 15s to extract the dansyl-Ala product and then centrifuged at 800g for 10min to separate the phases.

For each set of reactions, appropriate enzyme and substrate blanks were prepared. To assure the specificity of the reaction, samples were also incubated in the presence or absence of the carboxypeptidase M inhibitor *Mergetpa*.

RESULTS AND DISCUSSION

B2 BK receptors but not B1 BK receptors are present on aortic cryosections of healthy pigs. For the purpose of autoradiographic [^{3}H]BK equilibrium binding studies it has previously been shown that it is necessary to use a wide range of protease inhibitors (indicated in method section) to prevent BK from degradation [10,11,12,13]. In the presence of these inhibitors in control tissues only minimal degradation was observed at 4°C during 2h ranging from 8 to 12% of the 3nM [^{3}H]BK utilized.

Specific [^{3}H]BK binding on healthy porcine aortic cryosections was saturable with high affinity (fig.1). The plots of two similar experiments indicated a single B2 BK binding site with a Kd of 1.6 ± 0.3 nM (mean ± S.E.M.) and a Bmax of 13.7 ± 2.5 fmol [^{3}H]BK specifically bound/mg protein (mean ± S.E.M.).

[^{3}H]BK receptor binding specificity was evaluated in displacement experiments using a saturating concentration of [^{3}H]BK (3nM) in the presence or absence of unlabelled B2 and B1 agonists (BK and des-Arg9-BK) or antagonists (Hoe 140 and des-Arg9-Leu8-BK) at 1μM concentration each. The B2 ligands were equally capable to displace total [^{3}H]BK binding to the maximal obtainable specific binding amounting to approximately 30% of the total radioactivity bound. In contrast no effects due to the presence of B1 agonist and antagonist were observed. This indicated that in tissue from thoracic aorta of a healthy animal only B2 BK receptors

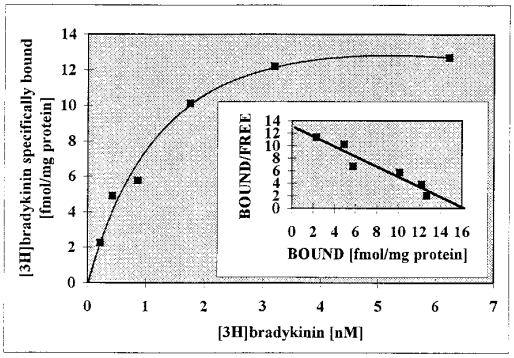

FIG. 1. Saturation curve and Scatchard analysis of [^{3}H]BK binding to cryosections from healthy porcine aortic tissue. 20μm cryosections were incubated at 4°C with increasing concentrations of [^{3}H]BK for 120min in the presence or absence of 1μM unlabelled BK. The binding reaction was terminated as described in Methods. Symbols (■) represent specific binding (total minus unspecific binding), in the inset Scatchard analysis of the binding results is depicted. Data points are best fitted by a straight line with a dissociation constant (Kd) of 1.3 nM and a maximal binding capacity (Bmax) of 16.2 fmol [^{3}H]BK specifically bound/mg protein. Data are from a representative experiment that was repeated once with similar results.

but not B1 receptors are being present in significant quantities.

Initial [^{3}H]BK binding studies in tissues derived from pigs with LPS infusion appeared to result in similar binding affinities and capacities (not shown). Surprisingly, however, in contrast to control tissues there was significant [^{3}H]BK degradation in incubations with cryosections of aorta obtained from a pig with LPS infusion even in the presence of the wide range of protease inhibitors and at low temperature (4°C). This resulted in non-equilibrium conditions preventing us from calculating correct [^{3}H]BK binding data for cryosections of aortae from pigs with LPS-induced inflammation.

B2 BK receptors are localized in the aortic endothelial layer. In initial binding studies we already observed that aortic tissue denuded from endothelium did not show detectable quantities of specific B2 receptor binding. These results indicated - by indirect means - that in intact aortic tissue B2 BK receptor binding sites are likely to be located within the endothelial cell layer. Our studies by microautoradiography revealed specific [^{3}H]BK binding sites above the endothelial cell layer of both aortic cryosections derived from a healthy piglet or from one with LPS-induced inflammation as evidenced by relating the emulsion autoradiograph to the histology of the cryosection. In fig.2 the results for tissue derived from a LPS-treated pig are depicted where most of the silver grains of the microautoradiogram are confined to the endothelium. The presence of the B2 BK receptor subtype on endothelium was also confirmed by using a multidomain B2 BK receptor subtype antibody (kindly provided from Dr. Müller-Esterl, Mainz, Germany) and subsequent peroxidase staining (not shown). This finding was expected since significant numbers of B2 BK receptors have previously been reported to occur in various endothelial preparations and endothelial cell cultures of different species [1]. Specifically in aortic endothelial cells BK, via B2 receptors, is known to induce arachidonic acid release [19] and to stimulate cGMP production [20], thereby participating in the regulation of blood vessel tonus and blood pressure.

Neither by autoradiographic visualization nor in displacement studies using B2 BK receptor ligands major differences in B2 BK receptor characteristics were observed between tissue obtained from healthy or LPS-treated piglets. These experiments are consistent with the hypothesis that experimentally induced sepsis is not causing a major difference in B2 BK receptor characteristics. Nonetheless in the same experimental model the *in vivo* application of B2 receptor antagonists has previously been shown to exert beneficial effects on survival of pigs with LPS shock [8].

Conversion of B2 receptor agonist to B1 receptor agonist in aortic cryosections derived from LPS-treated piglets. In initial studies on aortic cryosections obtained from a LPS-treated pig where we displaced [^{3}H]BK B2 receptor binding by B2 and B1 receptor agonists and antagonists, we observed significant competition not only by B2 ligands but surprisingly also by B1 receptor ligands (des-Arg9-BK, des-Arg9-Leu8-BK). This observation could only be explained by the hypothesis that during sepsis B1 BK receptors, not being detectable in control tissues, are induced and/or upregulated in the aortic vessel wall. At the same time this provided indirect evidence that [^{3}H]BK must - at least in part - be converted by a tissue carboxypeptidase activity to [^{3}H]-des-Arg9-BK in order to allow for displacement by B1 agonist or antagonist. This finding pointed also to a significantly enhanced carboxypeptidase activity during LPS treatment considering the fact that this phenomenon was observed only during inflammatory conditions and occurred even at 4°C and in the presence of an already wide range of protease inhibitors.

In evaluating the protease inhibitor cocktail we initially used it seemed as if there were no sufficient quantities of carboxypeptidase specific inhibitors. We have used 1,10-phenanthroline, an inhibitor of metalloproteases, at a concentration of 1μM since at higher concentrations this compound is directly affecting [^{3}H]BK binding (Faussner, unpublished results). In some studies [21], however, up to 2.5mM of the inhibitor is reported to be needed in order to fully prevent BK degradation by carboxypeptidases. Therefore, in view of the results in displacement studies, we speculated that in aortic tissues derived from a pig with LPS infusion a high carboxypeptidase activity is not fully inhibited by the inhibitors being utilized.

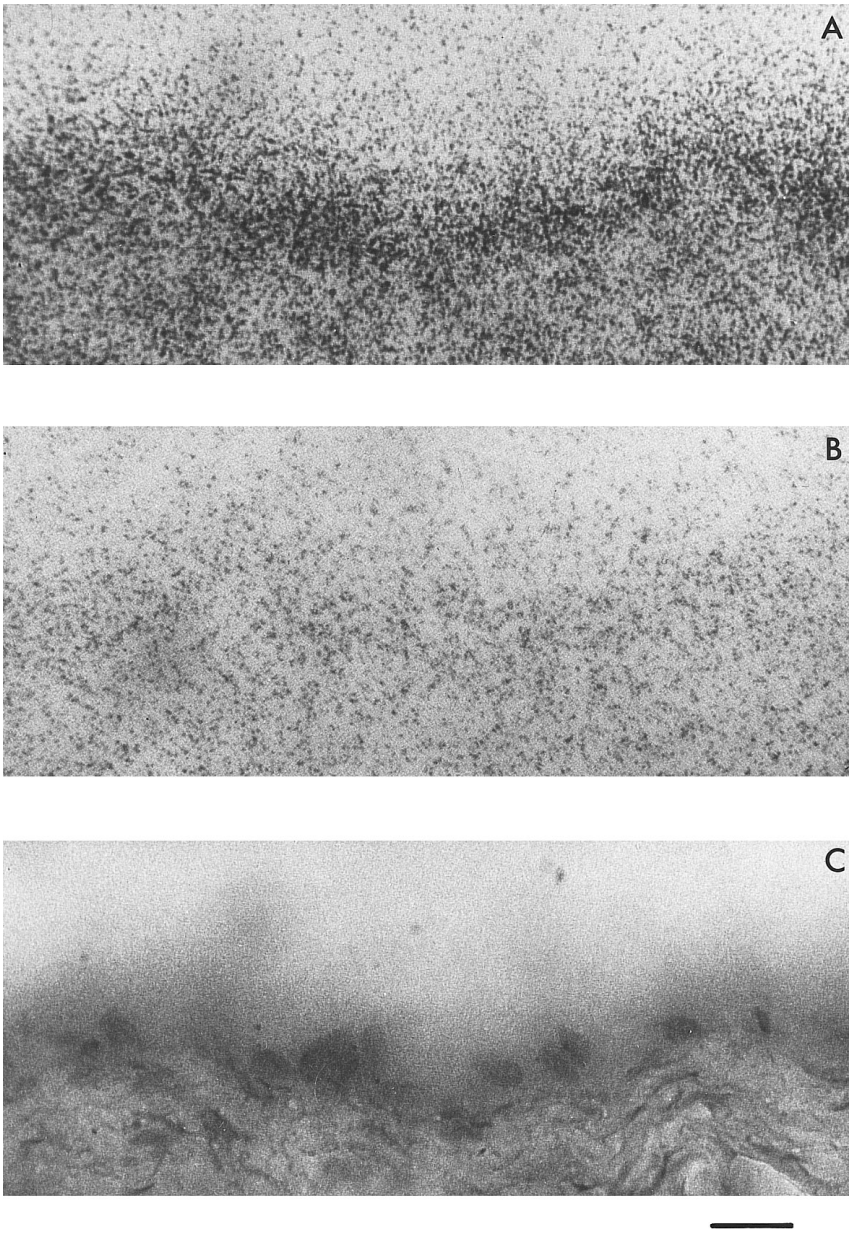

FIG. 2. Localization of [^{3}H]BK binding sites by microautoradiography in aortic cryosections derived from a LPS-treated pig. Emulsion-coated coverslips were used as previously described [12] for high resolution receptor microautoradiography. (A) Microautoradiograph of total binding (= incubation with 3.8nM [^{3}H]BK) shows intense labelling above the endothelium with much fewer, unspecific silver grains above the smooth muscle layer. (B) Microautoradiograph of nonspecific binding (= incubation with 3.8nM [^{3}H]BK in the presence of 1μM unlabelled BK). Only a few silver grains are scattered throughout the cryosection (comprising endothelium and smooth muscle layer) without any distinct preference for a certain cell layer. (C) Toluidine-blue-stained cryostat section depicting the nuclei of the endothelial layer. Length of calibration bar is 10μm.

To prove this hypothesis aortic cryosections from LPS-treated pigs where incubated with the B2 ligand [^{3}H]BK (5.8nM) in the presence of increasing concentrations of unlabelled des-Arg9-BK (fig.3). The experiment was performed in the presence or absence of the specific carboxypeptidase M inhibitor *Mergetpa* under the assumption that *Mergetpa* is capable to prevent conversion of [^{3}H]BK to [^{3}H]des-Arg9-BK. In fact an effective competition with des-Arg9-BK was only observed, provided that *Mergetpa* was omitted from the incubation buffer. Under these conditions 1μM des-Arg9-BK was able to displace specific [^{3}H]binding to

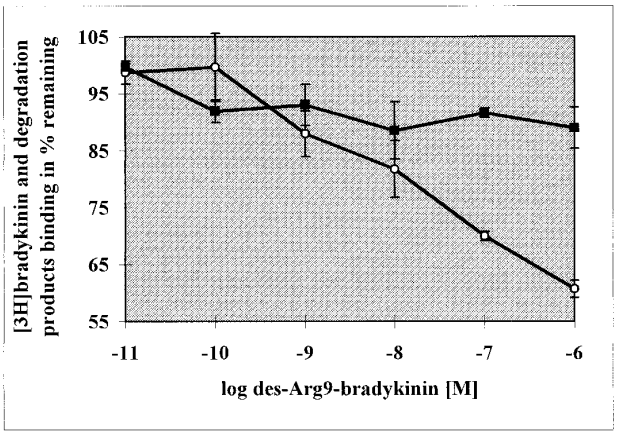

FIG. 3. Competition of [^{3}H]binding by des-Arg9-BK in aortic cryosections derived from a LPS-treated pig. Effect of the carboxypeptidase M inhibitor *Mergetpa*. Symbols represent % [^{3}H]-radioactivity remaining at the different concentrations of des-Arg9-BK in the absence (○) or presence (■) of 4.5mM *Mergetpa* (mean ± SD of triplicate determinations). The binding study was carried out by incubation with 3.8nM [^{3}H]BK as described in Methods. [^{3}H]BK binding is likely to be composed of both [^{3}H]BK and [^{3}H]des-Arg9-BK (in the absence of *Mergetpa*).

approximately 42% of control binding in both either sections from pigs with pre-existing inflammation or from those having received LPS-treatment.

This provided further indirect evidence of conversion of [^{3}H]BK to [^{3}H]des-Arg9-BK by a carboxypeptidase in cryosections derived from inflammed tissue.

A membrane bound carboxypeptidase M was suggested being responsible for this phenomenon since blood plasma derived carboxypeptidase N [15] obviously cannot be present during our *in vitro* incubations.

Endothelial carboxypeptidase M activity is upregulated during experimentally treatment with bacterial LPS and pre-existing inflammatory disease. Subsequently we directly measured the activity of carboxypeptidase M with dansyl-Ala-Arg as substrate in aortic tissues obtained from piglets during the different experimental conditions (fig.4). A marked upregulation of carboxypeptidase M activity reaching to 255 ± 8% (5.572 ± 0.446 nmoles dansyl-Ala/min/mg protein) in tissue derived from a piglet with pre-existing disease and up to 233 ± 12% (5.224 ± 0.623 nmoles dansyl-Ala/min/mg protein) for tissue derived from a piglet with experimentally induced LPS shock was observed. In cryosections prepared from de-endothelialized aortic tissue carboxypeptidase M activity was only minor and not influenced by the inflammatory conditions. This is consistent with the hypothesis that the enzyme activity is confined to the aortic endothelial cells.

It is tempting to speculate that upregulation of endothelial carboxypeptidase M activity is a general consequence of inflammatory processes. E.g. [22] reported that the level of a BK and kallidin degrading carboxypeptidase activity was significantly increased after allergen challenge of allergic, but not of nonallergic individuals. An increase of carboxypeptidase M activity has also been shown in bronchoalveolar lavage fluid in human lung diseases (infection with certain pathogens) by [23], and [24] showed that membrane fractions from cultured human pulmonary arterial endothelial cells contained high carboxypeptidase M activity.

B1 BK receptors are upregulated in the aortic smooth muscle layer during experimentally treatment with bacterial LPS and pre-existing inflammatory disease. We have previously shown by virtue of microautoradiography that in porcine pulmonary arteries the B1 BK receptors, being upregulated during LPS-induced shock and naturally occurring inflammatory processes, are localized within the muscle layer of the vessel [13]. In this study we were able to confirm this localization also for porcine thoracic aorta since very similar pictures were obtained by micoautoradiography (not shown). The localization of B1 receptors within the smooth muscle layer was also indirectly confirmed when we compared intact vessels with those being denuded from endothelium in B1 receptor binding studies using the specific radioligand [^{3}H]des-Arg10-kallidin (fig.5).

Whereas on cryostat sections from healthy pigs B1 receptor binding was barely detectable a dramatic increase of B1 receptor sites was seen both on sections derived from LPS-treated pigs (20- to 22-fold of control) and in sections obtained from pigs with pre-existing inflammation (24- to 26-fold of control). In displace-

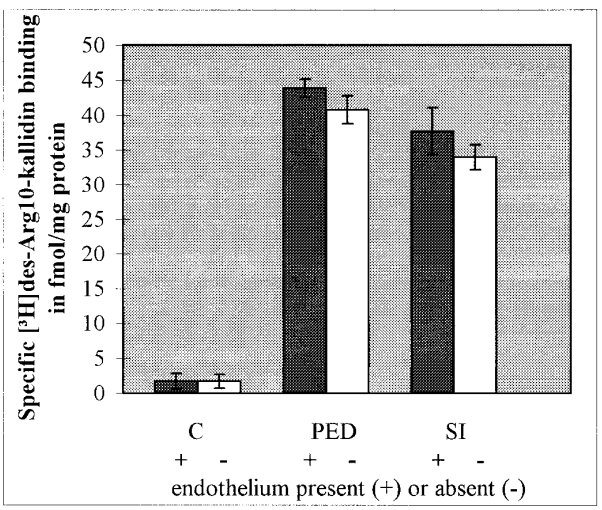

FIG. 4. Carboxypeptidase M activity of porcine aortic cryosections ± endothelium. Tissue was obtained from healthy control animal (C) or a pig with LPS-induced inflammation (SI) and a pig suffering from pre-existing inflammatory disease (PED). Carboxypeptidase M activity was evaluated according to Methods; values are given as means ± SD of triplicate determinations.

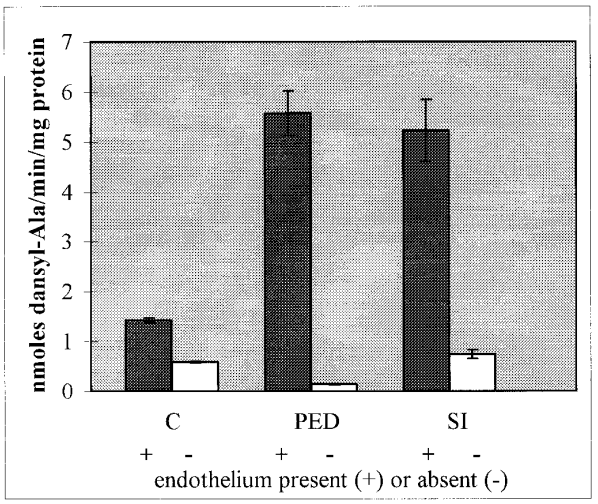

FIG. 5. Specific [^{3}H]des-Arg10-kallidin binding to cryosections of porcine aorta tissue obtained from healthy control animal (C) or a pig with LPS shock (SI) and a pig suffering from pre-existing inflammatory disease (PED). 20μm cryosections were incubated at 4°C with a saturating concentration of [^{3}H]des-Arg10-kallidin (5.8nM) for 120min in the presence (nonspecific binding) or absence (total binding) of 1μM unlabelled des-Arg10-kallidin. The binding reaction was terminated as described in Methods. Specific binding represents the difference between total binding and nonspecific binding.

ment studies B1 receptor agonists (des-Arg9-BK, des-Arg10-kallidin) and B1 receptor antagonist (des-Arg9-Leu8-BK) were equally effective in competing for [^{3}H]-des-Arg10-kallidin binding whereas the B2 antagonist HOE 140 showed no significant effect (not shown). These experiments confirmed the B1 subtype specificity of the receptors being upregulated during LPS treatment and pre-existing inflammatory disease. Under no condition significant differences due to the presence or absence of endothelium were observed. This is in contrast to the situation we noted in B2 receptor binding studies where de-endothelialized sections lost their capacity for binding the B2 receptor ligand [^{3}H]BK.

The exact localization of B1 BK receptors, upregulated within large blood vessels during LPS-induced inflammation, has not been known so far. Both morphological studies and direct receptor binding data clearly demonstrate that B1 BK receptors are confined mainly to the vascular smooth muscle layer. In contrast to B1 BK receptors B2 BK receptors are expressed constitutively on vascular endothelial cells. Their amount and affinity seems to be the same in vascular tissue derived from normal and LPS-treated animals. Nonetheless in vivo B2 antagonists appear to be protective in this LPS shock model, an effect that is reversed by simultaneous B1 receptor blockade [8,9].

We provide further evidence that not only B1 BK receptors but also carboxypeptidase M, generating the appropriate B1 receptor ligands, is simultaneously upregulated during LPS infusion or as result of pre-existing inflammatory processes.

An interesting observation of our study was, that a (local) pre-existing inflammation was inducing similar effects in extent than a more generalized bacteremia induced by LPS. It was repeatedly been shown that the local cytokine network, in particular interleukin-1, plays a crucial role in the mechanism of B1 receptor upregulation [5,25]. This further supports hypothesis that inflammatory mediators, like interleukin-1, may have an obligatory sensitizing role in the upregulation phenomenon.

Upregulated carboxypeptidase activity is most likely confined to the endothelium where the conversion from the naturally occurring B2 ligands BK and kallidin to the corresponding B1 ligands takes place. During inflammatory conditions in a crosstalk between endothelial cells and smooth muscle cells, where B1 receptors are located, the binding of the B1 ligand may generate functional responses that are needed to counteract some of the detrimental effects of inflammation.

ACKNOWLEDGMENTS

We thank R. A. Skidgel for kindly providing us with dansyl-Ala-Arg, B. Schoelkens for the gift of HOE 140, and W. Müller-Esterl for the provision of B2 BK receptor antibody. This work was supported by the Sonderforschungsbereich 207, Project F4, Ludwig-Maximilians-Universität, Munich.

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