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Lipopolysaccharide enhances bradykinin-induced signal transduction *via* activation of Ras/Raf/MEK/MAPK in canine tracheal smooth muscle cells

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1 Bacterial lipopolysaccharide (LPS) was found to induce inflammatory responses and to enhance bronchial hyperreactivity to several contractile agonists. However, the implication of LPS in the pathogenesis of bronchial hyperreactivity was not completely understood. Therefore, in this study, we investigated the effect of LPS on mitogen-activated protein kinase (MAPK) activation associated with potentiation of bradykinin (BK)-induced inositol phosphates (IPs) accumulation and Ca^{2+} mobilization in canine cultured tracheal smooth muscle cells (TSMCs).

2 LPS stimulated phosphorylation of p42/p44 MAPK in a time- and concentration-dependent manner using a Western blot analysis against a specific phosphorylated form of MAPK antibody. Maximal stimulation of the p42 and p44 MAPK isoforms occurred after 7 min-incubation and the maximal effect was achieved with 100 μg ml^{-1} LPS.

3 Pretreatment of TSMCs with LPS potentiated BK-induced IPs accumulation and Ca^{2+} mobilization. However, there was no effect on the IPs response induced by endothelin-1, 5-hydroxytryptamine, and carbachol. In addition, pretreatment with PDGF-BB enhanced BK-induced IPs response.

4 These enhancements by LPS and PDGF-BB might be due to an increase in BK B_2 receptor density (B_{max}) in TSMCs, characterized by competitive inhibition of [^{3}H]-BK binding using B_1 and B_2 receptor-selective reagents.

5 The enhancing effects of LPS and PDGF-BB were attenuated by PD98059, an inhibitor of MAPK kinase (MEK), suggesting that the effect of LPS may share a common signalling pathway with PDGF-BB in TSMCs.

6 Furthermore, overexpression of dominant negative mutants, H-Ras-15A and Raf-N4, significantly suppressed p42/p44 MAPK activation induced by LPS and PDGF-BB, indicating that Ras and Raf may be required for activation of these kinases.

7 These results suggest that the augmentation of BK-induced responses produced by LPS might be, at least in part, mediated through activation of Ras/Raf/MEK/MAPK pathway in TSMCs.
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Abbreviations: BK, bradykinin; B_{max}, total receptor density; BCA, bicinchoninic acid; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, concentration required for half-maximal stimulation; ECL, enhanced chemiluminescence; ET-1, endothelin-1; F-12, Ham's nutrient mixture F-12; FBS, foetal bovine serum; 5-HT, 5-hydroxytryptamine; IPs, inositol phosphates; KHS, Krebs-Henseleit buffer; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase; PDGF-BB, PDGF B-chain homodimer; PI, phosphoinositide; TSMCs, tracheal smooth muscle cells

Introduction

Lipopolysaccharides (LPS) are pro-inflammatory substances present in the cell wall of gram-negative bacteria. Besides infection, airborne bacteria are present in house dust (HD). Indeed, LPS is present in a wide variety of occupational and general environments (Rylander *et al*., 1985; Olenchock *et al*., 1987; Thelin *et al*., 1984). Several studies have demonstrated that severity of asthma is related to LPS in HD, suggesting that LPS is a triggering factor for increasing the severity of asthma (Platts-Mills & De Weck, 1989; Michel *et al*., 1996; Kline *et al*., 1999). LPS has been shown to be the most potent agent known to induce changes in mononuclear phagocyte function. Treatment of mononuclear phagocytes with LPS shows increased microbicidal activity, increased synthesis and release of inflammatory mediators such as interleukin-1 (IL-1), IL-6, tumour necrosis factor-α (TNF-α), PAF, and metabolites of arachidonic acid (Nathan, 1987; Morrison & Ryan, 1987). In addition, inhaled LPS can induce inflammatory responses in the respiratory system, such as an increase in microvascular permeability in bronchial and pulmonary circulation (Tamaoki *et al*., 1992) and recruitment of neutrophils into the air space (Hudson *et al*., 1977; Michel *et al*., 1989; 1992; Sandstrom *et al*., 1992). Thus, exposure to LPS is an important factor in the pathogenesis of aggravation of asthma and airway hyperresponsiveness (Michel *et al*., 1989; 1992; Hunt *et al*., 1994; Rylander *et al*., 1989; Sandstrom *et al*., 1992; Kline *et al*.,

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1999). However, the molecular mechanisms of LPS enhancing severity of asthma are not completely understood.

It has been shown that CD14, which is one of the glycosylphosphatidylinositol-anchored proteins, can mediate the LPS signal (Chaby & Girard, 1993). Several lines of evidence have indicated that the signalling pathways involve rapid activation of src family tyrosine kinases leading to the phosphorylation of tyrosine residue on certain target proteins (Weinstein *et al*., 1993; Stefanova *et al*., 1993). Among the most prominent tyrosine-phosphorylated proteins in LPS-stimulated macrophages are the p42 and p44 isoforms of mitogen-activated protein kinase (MAPK) (Weinstein *et al*., 1992; Ding *et al*., 1993; Dong *et al*., 1993; Larrivee *et al*., 1998). MAPKs are a family of serine/threonine protein kinases that participate in signalling pathways initiated by many extracellular stimuli. One potential signalling pathway activated by LPS may be the MAPK pathway identified as a downstream target of Ras proteins. The pathway is initiated by many different ligands, particularly polypeptide growth factors that activate intrinsic or extrinsic protein tyrosine kinase activity (Howe *et al*., 1992; Moodie *et al*., 1993). Moreover, Raf-1, which is able to interact directly with Ras, is an important intermediate along the pathway to MAPK activation (Howe *et al*., 1992; Moodie *et al*., 1993; Brtva *et al*., 1995). Raf-1 may participate in the activation of MAPK *via* both Ras-dependent and -independent mechanisms in macrophages (Buscher *et al*., 1995). Utilizing selective expression of dominant negative mutants, Ras, Raf, or MEK, has been shown to play a key step toward MAPK activation (Brtva *et al*., 1995; Chen *et al*., 1994; Abdellatif *et al*., 1998; Schaap *et al*., 1993). Moreover, LPS has been shown to cause a rapid phosphorylation and activation of Raf-1 in macrophages, suggesting that activation of these proteins occurs downstream of tyrosine phosphorylation (Reimann *et al*., 1994). These findings further imply the role of these MAPK pathways as important signalling mechanisms underlying the LPS-induced airway hyperreactivity.

In addition, several studies demonstrate that responses to LPS in a variety of cell types may involve a pertussis toxin-sensitive G protein (Jakway & DeFranco, 1986; Dziarski, 1989; Wang *et al*., 1988), activation of phospholipase C (PLC), increase of intracellular Ca^{2+} concentration (Waga *et al*., 1993), and activation of protein tyrosine kinases (Han *et al*., 1994), and protein kinase C (PKC) (Fujihara *et al*., 1994). The cascade reaction of these kinases leads to the activation and the induction of gene expression. LPS has been shown to up-regulate the expression of PDGF receptors in rat lung myofibroblasts and to enhance PDGF responses (Coin *et al*., 1996) and to enhance the expression of BK receptors in the rat nephron (Marin-Castano *et al*., 1998). Up to date, it is not known whether LPS may alter the affinity and/or the number of receptors related to stimulus-response coupling in canine tracheal smooth muscle.

In addressing these questions, experiments were undertaken to investigate the effect of LPS on activation of Ras/Raf/MEK/MAPK pathway and the mechanisms underlying the enhancement by LPS on IPs accumulation and Ca^{2+} mobilization induced by contractile agonists. Our results show that LPS stimulates phosphorylation of p42/p44 MAPK in TSMCs. Pretreatment with LPS and PDGF-BB greatly enhances the BK-induced responses in these cells. Finally, these results demonstrate that the increased responsiveness of the TSMCs induced by LPS results, at least in part, from an increase in the number of BK receptors through activation of Ras/Raf/MEK/MAPK pathway.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium, OPTI-MEM I medium, Lipofectamine Plus reagent, and foetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). Myo-[^{3}H]-inositol (18 Ci mmol^{-1}), [^{3}H]-BK (71 Ci mmol^{-1}), Hybond C membrane, and enhanced chemiluminescence (ECL) Western blotting detection system were from Amersham (Buckinghamshire, U.K.). Fura-2/AM was from Molecular Probes (Eugene, OR, U.S.A.). PhosphoPlus MEK1/2 and PhosphoPlus p42/p44 MAPK antibody kits were from New England Biolabs (Beverly, MA, U.S.A.). ERK1 (p44 MAPK) and ERK2 (p42 MAPK) antibodies were from Santa Cruz (Santa Cruz, CA, U.S.A.). PD98059 and SB203580 were from Calbiochem (San Diego, CA, U.S.A.). Recombinant human PDGF-BB was from Pepro Tech (London, U.K.). Bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL, U.S.A.). Enzymes and other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Animals

Mongrel dogs of either sex, 10–20 kg, purchased from a local supplier were used throughout this study. Dogs were housed indoors in the animal facilities under automatically controlled temperature and light cycle and fed standard laboratory chow and tap water *ad libitum*. Dogs were anaesthetized with ketamine (20 mg kg^{-1}, w v^{-1}, i.m.) and pentobarbitone (30 mg kg^{-1}, w v^{-1}, intravenously) and the lungs were ventilated mechanically *via* an orotracheal tube. The tracheas were surgically removed.

Isolation of tracheal smooth muscle cells

The TSMCs were isolated according to the methods as described previously (Yang *et al*., 1994a,b; 1995). Briefly, the muscle was dissected, minced and transferred to the dissociation medium containing 0.2% (w v^{-1}) collagenase I, 0.01% (w v^{-1}) deoxyribonuclease I, 0.01% (w v^{-1}) elastase IV, and antibiotics in a physiological solution. The released cells were collected and the residual was again digested with fresh enzyme solution for an additional 1 h at 37°C. The cell number was counted and the suspension diluted with DMEM/F-12 containing 10% FBS to a concentration of 2×10^5 cells ml^{-1}. The cell suspension was plated onto (0.5 ml per well) 24-well, (1 ml per well) 12-well, and (2 ml per well) 6-well culture plates containing glass coverslips coated with collagen for receptor binding assay, IPs accumulation, and $[Ca^{2+}]_i$ measurement, respectively. Culture medium was changed after 24 h and then every 3 days.

In order to characterize the isolated and cultured TSMCs and to exclude contamination by epithelial cells and fibroblasts, the cells were identified by an indirect immunofluorescent staining method using a monoclonal antibody of light chain myosin (Gown *et al*., 1985). Over 95% of the cell preparation was found to be composed of smooth muscle cells.

Accumulation of inositol phosphates

Effect of BK on the hydrolysis of phosphoinositide (PI) was assayed by monitoring the accumulation of [^{3}H]-labelled IPs as described by Yang *et al*. (1994a). Cultured TSMCs were

incubated with 5 μCi ml^{-1} of myo-[2-^{3}H]-inositol at 37°C for 48 h. The cells were treated with LPS or PDGF-BB for the appropriate time during the myo-[^{3}H]-inositol labelling period. When inhibitors were used, they were added 1 h prior to the application of LPS or PDGF-BB. TSMCs were washed twice with PBS and incubated in Krebs-Henseleit buffer (KHS, pH 7.4) containing (in mM) NaCl 117, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.2, $NaHCO_3$ 20, $CaCl_2$ 2.4, glucose 1, HEPES 20, and LiCl 10 at 37°C for 30 min. After BK added at the concentration indicated, incubation was continued for another 60 min. Reactions were terminated by addition of 5% (w v^{-1}) perchloric acid followed by sonication and centrifugation at $3000 \times g$ for 15 min.

The perchloric acid soluble supernatants were extracted four times with ether, neutralized with potassium hydroxide, and applied to a column of AG1-X8, formate form, 100–200 mesh (Bio-Rad, Hercules, CA, U.S.A.). The resin was washed successively with 5 ml of water and 5 ml of 60 mM ammonium formate-5 mM sodium tetraborate to eliminate free myo-[^{3}H]-inositol and glycerophosphoinositol, respectively. The fraction of total IPs was eluted with 5 ml of 1 M ammonium formate-0.1 M formic acid. The amount of [^{3}H]-IPs was determined in a radiospectrometer (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Measurement of intracellular Ca^{2+} level

$[Ca^{2+}]_i$ was measured in confluent monolayers with the calcium-sensitive dye fura-2/AM as described by Grynkiewicz *et al.* (1985). Upon confluence, the cells were cultured in DMEM/F-12 with LPS for 24 h before measurements were made. When inhibitors were used, they were added 1 h prior to the application of LPS. The monolayers were covered with 1 ml of DMEM/F-12 with 1% FBS containing 5 μM fura-2/AM and were incubated at 37°C for 45 min. At the end of the period, the coverslips were washed twice with the physiological buffer solution containing (mM): NaCl 125, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 2, NaH_2PO_4 0.5, $NaHCO_3$ 5, HEPES 10, glucose 10, pH 7.4. The cells were incubated in PBS for further 30 min to complete dye de-esterification. The coverslip was inserted into a quartz cuvette at an angle of approximately 45° to the excitation beam and placed in the temperature-controlled holder of a Hitachi F-4500 spectrofluorometer (Tokyo, Japan). Continuous stirring was achieved with a magnetic stirrer. Fluorescence of Ca^{2+}-bound and unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths between 340 and 380 nm and electronically separating the resultant fluorescence signals at emission wavelength 510 nm. The autofluorescence of each monolayer was subtracted from the fluorescence data. The ratios (R) of the fluorescence at the two wavelengths are computed and used to calculate changes in $[Ca^{2+}]_i$. The ratios of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were determined by adding ionomycin (10 μM) in the presence of PBS containing 5 mM Ca^{2+} and by adding 5 mM EGTA at pH 8 in a Ca^{2+}-free PBS, respectively. The K_d of fura-2 for Ca^{2+} was assumed to be 224 nM (Grynkiewicz *et al.*, 1985).

[^{3}H]-BK binding assay

For detecting the effects of LPS and PDGF-BB on BK receptor density or affinity of TSMCs, [^{3}H]-BK was used as a radioligand. Binding assays were performed with confluent TSMCs on 24-well culture plates, treated with or without LPS or PDGF-BB treatment in DMEM/F-12 for 24 h prior to the binding experiments, as previously described (Yang *et al.*, 1995). When inhibitors were used, they were added 1 h prior to the application of LPS or PDGF-BB. Total receptor density (B_{max}) and dissociation constant (K_D) were calculated by Prizm program, as described previously (Yang *et al.*, 1995). Half-maximal inhibitory concentration (IC_{50}) values were calculated from competition experiments by Prizm program. IC_{50} values were transformed to apparent inhibitory constant (K_i) values. Subtypes analysis was performed by fitting the competitive inhibition curves with either a one- or a two-binding site model using an iterative least-squares fit by Prizm program that corrected for occupancy of [^{3}H]-BK with statistical significance established by Fisher's *F*-test. Protein concentration was measured by the method of Bradford (1976).

Plasmids and transfection

The plasmids encoding H-Ras-15A and Raf-N4 (dominant negative mutants of Ras and Raf-1), cloned into pZIP-NeoSV and pCGN, respectively, were kindly provided by Dr Channing J. Der (Department of Pharmacology, University of North Carolina at Chapel Hill). All plasmids were prepared by using QIAGEN plasmid DNA preparation kits.

TSMCs were plated at 3×10^5 cells ml^{-1} (2 ml per well) in 6-well culture plates for 24 h, reaching about 80% confluence. Cells were washed once with PBS and once with serum-free DMEM/F-12, and 0.8 ml of serum-free OPTI-MEM I medium was added to each well. The DNA PLUS-Lipofectamine reagent complex was prepared according to the instructions of manufacturer (GIBCO-BRL). The amount of transfected plasmid was kept constant (2 μg of H-Ras-15A, Raf-N4, pZIP-NeoSV, and pCGN, for each well). The DNA PLUS-Lipofectamine reagent complex (0.2 ml) was added to each well, then incubated at 37°C for 5 h, at that time 1 ml of OPTI-MEM I medium containing 20% FBS was added and incubated for 19 h. After 24 h of transfection, the cells were washed twice with PBS and maintained in DMEM/F-12 containing 10% FBS for 48 h. Cells were then washed once with PBS and incubated with serum-free DMEM/F-12 for 24 h before treatment with either LPS or PDGF-BB.

Preparation of cell extracts and Western blot analysis of MAPK isoforms

For experiments, cells were plated in 100-mm dishes and made quiescent at confluence by incubation in serum-free DMEM/F-12 for 24 h. Growth-arrested TSMCs were incubated with or without LPS at 37°C for various times. When inhibitors were used, they were added 1 h prior to the application of LPS. After incubation, the cells were then rapidly washed with ice-cold PBS, scraped and collected by centrifugation at $1000 \times g$ for 10 min. The collected cells were lysed with ice-cold lysis buffer containing (mM): Tris-HCl 25, pH 7.4, NaCl 25, NaF 25, sodium pyrophosphate 25, sodium vanadate 1, EDTA 2.5, EGTA 2.5, Triton X-100 0.05% (w v^{-1}), SDS 0.5% (w v^{-1}), deoxycholate 0.5% (w v^{-1}), NP-40 0.5% (w v^{-1}), leupeptin 5 μg ml^{-1}, aprotinin 5 μg ml^{-1}, and PMSF 1. The lysates were centrifuged at $45{,}000 \times g$ for 1 h at 4°C to yield the whole cell extract. The protein concentration was determined by the BCA reagents according to the instructions of the manufacturer. Samples from these supernatant fractions (30 μg protein) were denatured and subjected to SDS–PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and the membrane was incubated successively at room temperature with 5% (w v^{-1}) BSA in TTBS (Tris-HCl 50 mM, NaCl 150 mM, 0.05% (w v^{-1}) Tween 20, pH 7.4) for 1 h. The phosphorylation of MEK1/2 or p42/p44 MAPK isoforms was

identified and quantified by Western blot analysis using Phosphoplus MEK1/2 and Phospho-p42/44 MAPK antibody kits according to the recommendation of the manufacturer. Briefly, membranes were incubated overnight at 4°C with the anti-phospho-MAPK polyclonal antibody used at a dilution of 1 : 1000 in TTBS. Membranes were washed with TTBS four times for 5 min each, incubated with a 1 : 1500 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. Following each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL (Amersham International).

Analysis of data

Concentration-effect curves were fitted and EC_{50} values were estimated using the Graph Pad Program (GraphPad, San Diego, CA, U.S.A). Data were expressed as the mean ± s.e.mean and analysed with a two-tailed Student's *t*-test at a $P<0.05$ level of significance.

Results

Effect of LPS on the phosphorylation of MAPK isoforms

MAPKs, a group of components in the signal transduction pathway, have shown to be activated by different stimuli in several cell types. Activation of p42 and p44 MAPK isoforms is accompanied by phosphorylation of the Tyr204 residue (Marshall, 1995). Therefore, we determined whether LPS activated MAPKs in TSMCs. When TSMCs were stimulated with LPS (100 μg ml^{-1}) for various times, densitometric analysis of the blot revealed that at 3 min, LPS induced a significant phosphorylation of both isoforms above control level and a maximal stimulation of the p42 and p44 MAPK isoforms occurred at 7 min and sustained for at least 2 h (Figure 1). Parallel blots run as controls that used antibody directed against the total p44 MAPK did not show any change (Figure 1). Furthermore, the LPS-induced phosphorylation of p42/p44 MAPK was concentration-dependent. Densitometric analysis of the blot indicated that the maximal effect was achieved with 100 μg ml^{-1} LPS (Figure 2).

Effects of MEK inhibitors on LPS-Induced MAPK phosphorylation

To ensure that the effect of LPS is mediated through the activation of tyrosine kinase/MAPK pathway, the effect of LPS on the MAPK phosphorylation was examined after treatment of TSMCs with 30 μM PD98059 (a synthetic MEK1/2 inhibitor), 30 μM SB203580 (a p38 MAPK inhibitor), or 30 μM genistein (an inhibitor of tyrosine kinase) for 1 h. As shown in Figure 3, the effects of these inhibitors on MAPK phosphorylation were assessed by Western blot analysis. The phosphorylation of p42/p44 MAPK induced by LPS was significantly inhibited by PD98059 and genistein, but not by SB203580, confirming that tyrosine kinase and MEK1/2 are required for MAPK activation in these cells. This hypothesis was further supported by the results that LPS-induced MEK1/2 activation in a time- and concentration-dependent manner (Figure 4). These data suggest that LPS-induced phosphorylation of p42/p44 MAPK was mediated through the activation of MEK1/2 in canine TSMCs.

Effect of BK on IPs accumulation

In the studies described above, exposure of TSMCs to LPS for 24 h significantly stimulated phosphorylation of p42/p44 MAPK that may associate with airway hyperreactivity. We further examined whether LPS can modulate the IPs accumulation induced by contractile agonists, TSMCs were pre-labelled with [^{3}H]-inositol for 48 h, treated with or without LPS (100 μg ml^{-1}) for 24 h, before addition of agonists. To define the time course by which LPS modulates BK-induced IPs response, TSMCs were treated with LPS for various times, and then the amount of IPs accumulation was compared with control. The LPS-enhanced the BK-induced IPs accumulation

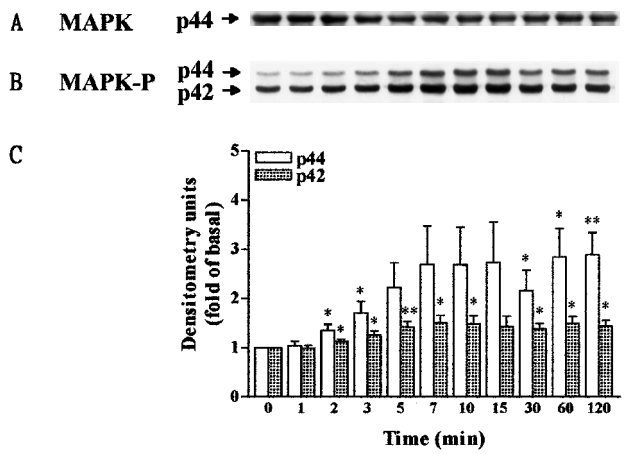

Figure 1 Time course of LPS-stimulated p42/p44 MAPK phosphorylation in TSMCs. The cells were grown to confluency, made quiescent by serum-deprivation for 24 h and incubated with 100 μg ml^{-1} LPS from 5–120 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with the total p44 MAPK (A) and anti-phospho-p42/p44 MAPK polyclonal antibody (B). Bands were visualized by an ECL method. Data are expressed as the mean ± s.e.mean of three independent experiments (C). *$P<0.05$; **$P<0.01$, as compared with the basal.

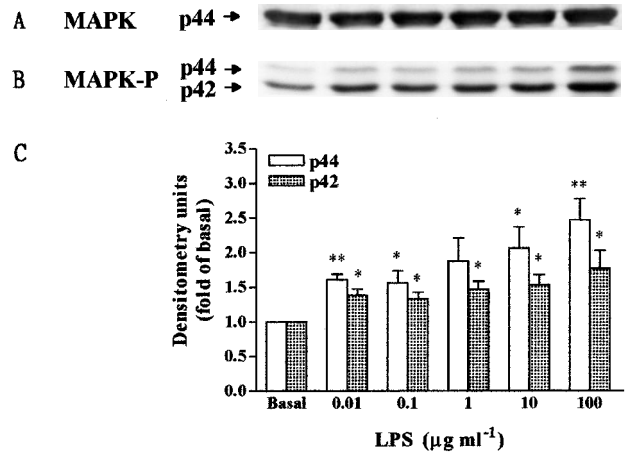

Figure 2 Concentration-dependence of LPS-stimulated p42/p44 MAPK phosphorylation in TSMCs. The cells were grown to confluence, made quiescent by serum-deprivation for 24 h and incubated with various concentrations of LPS for 15 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method directed against with the total p44 MAPK (A) and anti-phospho-p42/p44 MAPK polyclonal antibody (B) as described in Figure 1. Data are expressed as the mean ± s.e.mean of three independent experiments (C). *$P<0.05$; **$P<0.01$, as compared with the basal.

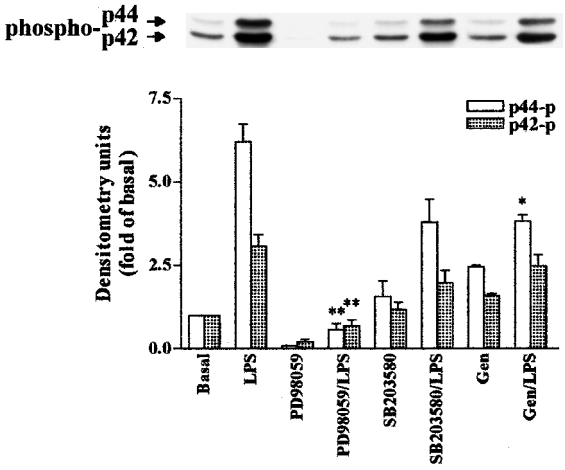

Figure 3 Effects of MAPK kinase inhibitors on LPS-stimulated p42/p44 MAPK phosphorylation in TSMCs. The cells were grown to confluence, made quiescent by serum-deprivation for 24 h. The cells were preincubated with 30 μM PD98059, 30 μM SB203580 or 30 μM genistein for 1 h, and then were stimulated with 100 μg ml^{-1} LPS for 15 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Bands were visualized by an ECL method as described in Figure 1. Similar results were obtained in three independent experiments. *$P<0.05$; **$P<0.01$, as compared with the cells stimulated with LPS alone.

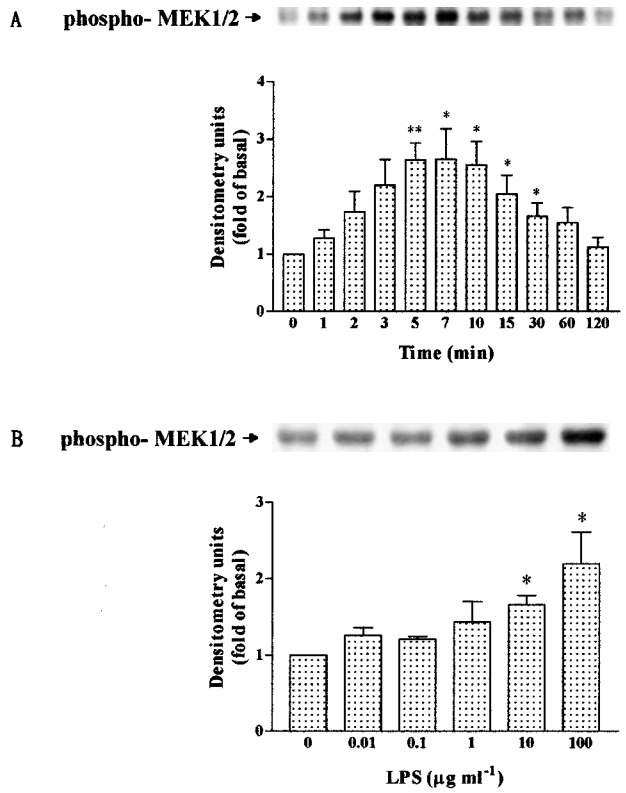

Figure 4 Time course and concentration-dependence of LPS-stimulated activation of MEK1/2 in TSMCs. The cells were grown to confluence, made quiescent by serum-deprivation for 24 h and incubated with 100 μg ml^{-1} LPS for various times (A) or various concentrations of LPS for 15 min (B). The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with anti-phospho-MEK1/2 polyclonal antibody. Bands were visualized by an ECL method and quantified by a densitometer. Similar results were obtained in three independent experiments. *$P<0.05$; **$P<0.01$, as compared with the basal.

was time-dependent (Figure 5A). The enhancement of the BK-induced IPs accumulation was not seen until 4 h of treatment, and a maximal enhancement apparently occurred after 24 h treatment with LPS (Figure 5A). In addition, LPS induced a concentration-dependent enhancement of BK response (Figure 5B). The half-maximal (EC_{50}) and maximal effects of LPS were obtained at concentrations of 5 and 100 μg ml^{-1}, respectively. Furthermore, the enhancement of IPs accumulation by

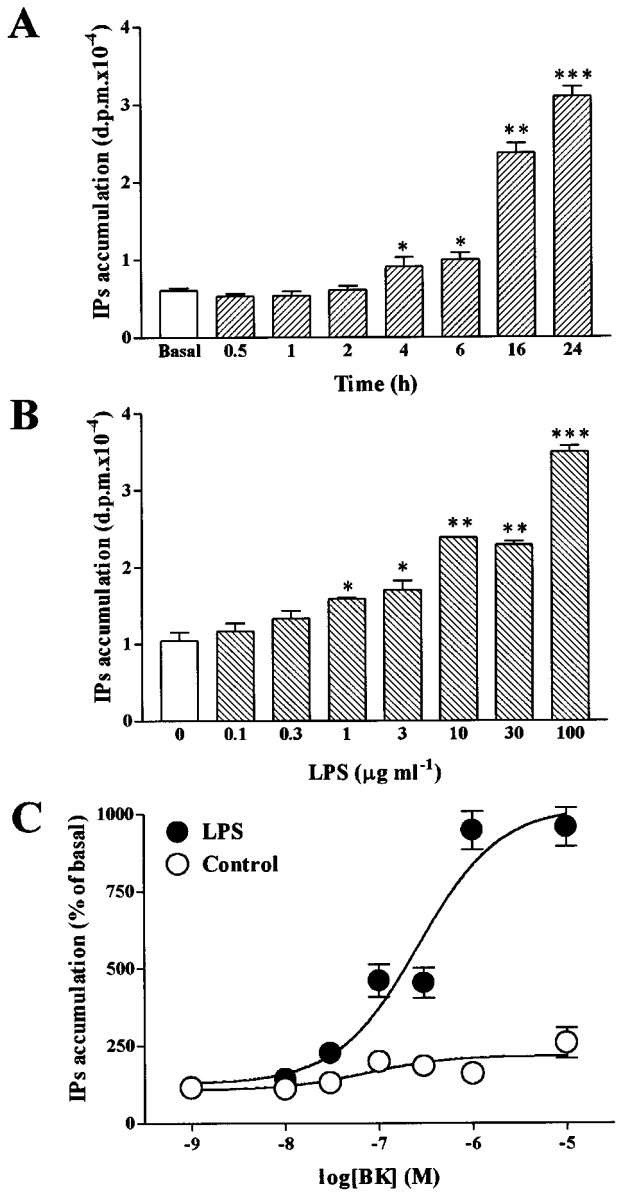

Figure 5 Effect of LPS treatment on BK-induced IPs accumulation in TSMCs. (A) Time-dependence of LPS augmentation of BK-stimulated IPs accumulation. Cells were pre-labelled with [^{3}H]-inositol for 48 h and incubated with either vehicle (basal) or 100 μg ml^{-1} LPS at 37°C for the indicated time, and then exposed to BK (10 μM) for 60 min. (B) Concentration-dependence of LPS augmentation of BK-stimulated IPs accumulation. [^{3}H]-inositol pre-labelled TSMCs were incubated with either vehicle (basal) or LPS at the concentrations indicated for 24 h, and then exposed to BK (10 μM) for 60 min. (C) Effect of LPS treatment on concentration-effect relationship for BK-induced IPs accumulation. [^{3}H]-inositol pre-labelled TSMCs were incubated with either vehicle (basal) or 100 μg ml^{-1} LPS for 24 h, and then exposed to various concentrations of BK for 60 min. The accumulation of IPs was determined, as described under Methods. Data are expressed as the mean ± s.e.mean of a single experiment performed in triplicate and representative of three separate experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$, as compared with control.

pretreatment with LPS was dependent upon the BK concentrations when measured 60 min after exposure to BK (Figure 5C). LPS increased the maximal BK-induced IPs accumulation, but did not change the basal level (2600±250 d.p.m. per well) when LPS was used alone. The concentration-effect relationship of BK-induced IPs accumulation was shifted to the left and BK was substantially more effective in TSMCs treated with LPS than in the control (Figure 5C). In contrast, B1 receptor-selective agonist des-Arg^9-BK did not cause any significant increase in IPs accumulation (data not shown).

To further characterize whether treatment of TSMCs with LPS also enhanced IPs accumulation induced by other contractile agonists, under similar conditions as described above, exposure of TSMCs to LPS for 24 h did not potentiate the IPs responses to endothelin-1 (ET-1, 1 μM), 5-hydroxytryptamine (5-HT, 100 μM), and carbachol (100 μM) (Figure 6). However, LPS selectively potentiated the IPs accumulation induced by BK. These results suggest that LPS might induce changes in some components unique to the BK receptor-coupling signalling transduction pathway in TSMCs.

Effect of BK on $[Ca^{2+}]_i$

Since LPS treatment increased the magnitude of BK-induced IPs accumulation, and it has been well established that generation of IP_3 leading to an increase of $[Ca^{2+}]_i$ is a predominant signalling mechanism which contributes to the contraction of airway smooth muscle (Yang *et al*., 1994b), we therefore evaluated the effect of LPS on the BK-induced increase in $[Ca^{2+}]_i$. As shown in Figure 7, LPS increased the maximal BK-induced rise in $[Ca^{2+}]_i$, but did not change the resting level of $[Ca^{2+}]_i$ (255±19 nM, $n=15$), similar to the observation obtained using macrophages (Hasko *et al*., 1998). The concentration-effect relationship of BK-induced increase in $[Ca^{2+}]_i$ was shifted to the left and BK was substantially more effective in TSMCs treated with LPS than in the control (Figure 7).

Effects of MEK inhibitors on LPS-enhanced responses to BK

To determine whether the enhancing effect of LPS on BK-induced responses was mediated through the activation of p42/p44 MAPK pathway, which shared this common pathway with growth factors, the effects of LPS and PDGF-BB on BK-induced IPs accumulation were measured in TSMCs pretreated with PD98059. As shown in Table 1, pretreatment of TSMCs with LPS or PDGF-BB for 24 h enhanced BK-induced IPs accumulation. Prior treatment of PD98059 (30 μM) significantly attenuated the enhancing effect of LPS or PDGF-BB on BK-induced IPs response. In a parallel experiments, treatment of TSMCs grown on collagen-coated glass coverslips with 30 μM PD98059 significantly attenuated the BK-induced increase in $[Ca^{2+}]_i$, as compared with LPS treated cells (Figure 8). These results suggest that the enhancing effect of LPS might be mediated through the activation of p42/p44 MAPK in canine TSMCs.

Effect of LPS on [^{3}H]-BK binding

LPS has been shown to enhance the expression of cell surface receptors and the synthesis of inflammatory mediators in several cell types (Marin-Castano *et al*., 1998; Coin *et al*., 1996;

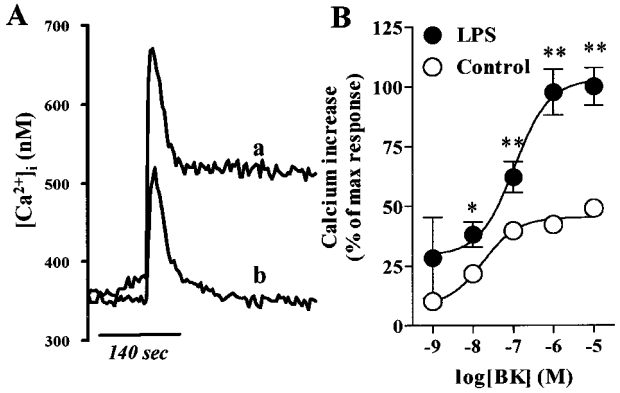

Figure 7 Effect of LPS treatment on concentration-effect relationship for BK-induced Ca^{2+} mobilization. TSMCs were incubated with either vehicle or 100 μg ml^{-1} LPS for 24 h and then exposed to various concentrations of BK. (A) The cells on glass coverslips were loaded with 5 μM fura-2/AM and fluorescent measurement of $[Ca^{2+}]_i$ was carried out in a dual excitation wavelength (340 and 380 nm) spectrofluorometer, (a, LPS; b, control; both of cell preparations were exposed to 10 μM BK). (B) Data are normalized to the maximal increase in the transient peak induced by BK (223±29 nM) above the resting levels in non-treated cells (270±11 nM) and expressed as the mean±s.e.mean of six experiments. *$P<0.05$; **$P<0.01$, as compared with the control.

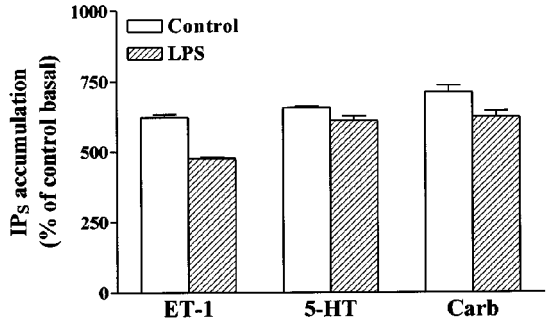

Figure 6 Effect of LPS treatment on IPs accumulation induced by various agonists. [^{3}H]-inositol prelabelled TSMCs were incubated with either vehicle (basal) or 100 μg ml^{-1} LPS at 37°C for 24 h, and then exposed to either ET-1 (1 μM), 5-HT (100 μM), or carbachol (100 μM) for 60 min. The accumulation of IPs was determined, as described under Methods. Data are normalized to the basal level of IPs accumulation and expressed as the mean±s.e.mean of six separate experiments performed in triplicate.

Table 1 Effects of LPS and PDGF-BB treatment on BK-induced accumulation of IPs in canine tracheal smooth muscle cells

	IP_s accumulation (d.p.m.well x 10^{-4})	
Treatment	*Basal*	*Bradykinin*
Control		
Untreated	0.26±0.02	0.91±0.05
LPS	0.32±0.01	5.65±0.25*
PDGF	0.30±0.02	2.51±0.19*
PD98059		
Untreated	0.28±0.02	0.66±0.05
LPS	0.24±0.01	0.91±0.05**
PDGF	0.32±0.01	0.41±0.02**

TSMCs were pre-labelled with [^{3}H]-inositol for 48 h, and treated with LPS (100 μg ml^{-1}) and PDGF-BB (20 ng ml^{-1}) in the absence or presence of PD98059 (30 μM) for 24 h, and then exposed to BK (10 μM) for 60 min. The results are expressed as the mean±s.e.mean of three separate experiments determined in triplicate. *$P<0.01$ as compared with untreated cells of control; **$P<0.01$ as compared with control cells stimulated by respective reagents.

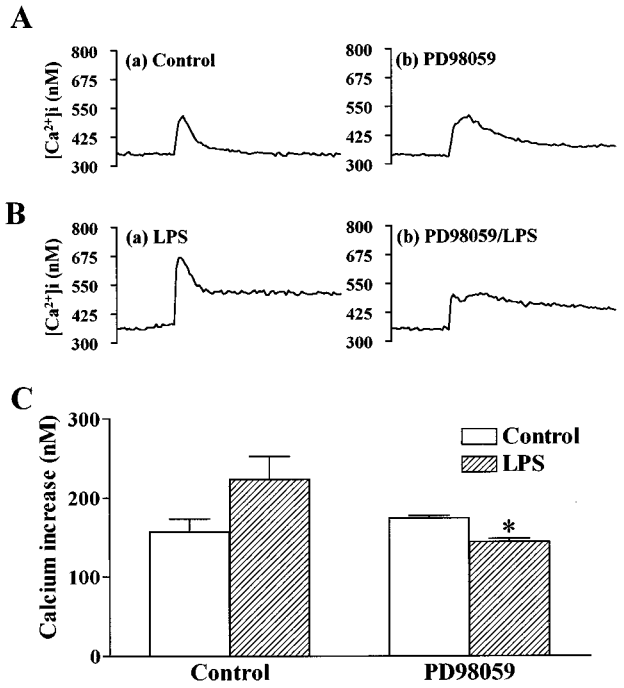

Figure 8 Effect of PD98059 on potentiating effect of LPS on BK-induced Ca^{2+} mobilization. Canine TSMCs were incubated in the absence (A) or presence (B) of 100 μg ml^{-1} LPS for 24 h, both in the absence (a) and presence (b) of PD98059. When PD98059 (30 μM) was used, it was added 1 h prior to the application of LPS. After treatment with LPS, TSMCs were loaded with 5 μM Fura-2/AM and stimulated with 10 μM BK. The change in $[Ca^{2+}]_i$ was measured as described under the Methods. Data are the net increase above the resting level of $[Ca^{2+}]_i$ (270$\pm$11 nM) and expressed as the mean$\pm$s.e.mean of six separate experiments, showed in (C). *$P<0.05$, as compared with the control.

Nathan, 1987; Morrison & Ryan, 1987). Based on our studies, it is evident that pretreatment of TSMCs with LPS enhances BK-induced accumulation of IPs and rise in $[Ca^{2+}]_i$. This might be due to changes in the affinity (dissociation constant, K_D) and/or an increase in the density of BK receptor (B_{max}). To determine whether the potentiation by LPS and PDGF-BB on BK-stimulated responses in TSMCs occurs at the level of BK receptors, we further measured B_{max} and K_D in cells treated with these two reagents for 24 h, using [^{3}H]-BK as a radioligand. As shown in Table 2, when TSMCs were cultured with LPS and PDGF-BB for 24 h, the B_{max} values significantly increased from 30$\pm$4 (non-treated cells) to 85$\pm$9 (LPS-treated cells) and 52$\pm$7 (PDGF-BB-treated cells) fmol mg^{-1} protein. These increases in [^{3}H]-BK binding sites induced by LPS and PDGF-BB were significantly reversed by concurrent incubation with PD98059. The K_D values were not significantly changed as compared with that of the control cells.

To examine further which BK receptor subtype was increased in the cells treated with LPS for 24 h, we chose des-Arg^9-BK, [Leu^8, des-Arg^9]-BK, BK, Lys-BK, [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK and Hoe 140 to differentiate between the BK binding sites. Data in Table 3 show that [^{3}H]-BK binding to TSMCs treated with LPS was inhibited by the B_2 receptor-selective agonists (BK and Lys-BK) and antagonists ([D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK and Hoe 140), but not affected by the B_1 receptor-selective agonist (des-Arg^9-BK) and antagonist ([Leu^8, des-Arg^9]-BK). Although the B_1 receptor-selective reagents inhibited [^{3}H]-BK binding by approximate 10% at the concentration of 100 μM, this concentration was far from their K_i values acting on B_1 receptors. These results suggest that the increase in receptor density is primarily due to the B_2 receptor.

Table 2 Effects of LPS and PDGF-BB treatment [^{3}H]-bradykinin ([^{3}H]-BK) binding in canine tracheal smooth muscle cells

Treatment	K_d (nM)	B_{max} (fmol mg^{-1} protein)
Control		
Untreated	0.92$\pm$0.17	30$\pm$4 (n=7)
LPS	0.76$\pm$0.19	85$\pm$9* (n=5)
PDGF	1.22$\pm$0.09	52$\pm$7* (n=5)
PD98059		
Untreated	1.06$\pm$0.34	30$\pm$13 (n=4)
LPS	0.78$\pm$0.22	50$\pm$12** (n=4)
PDGF	1.07$\pm$0.17	19$\pm$12** (n=3)

Cultured TSMCs were incubated in the absence or presence of PD98059 (30 μM) with either LPS (100 μg ml^{-1}) of PDGF-BB (20 ng ml^{-1}) for 24 h. Binding assays were performed in triplicate with concentrations of [^{3}H]-BK ranging from 0.2 to 6 nM and incubated at 4°C for 4 h. Data are expressed as the mean$\pm$s.e.mean of at least three individual experiments (n). *$P<0.01$ as compared with control untreated cells, **$P<0.01$ as compared with control cells treated with LPS or PDGF.

Table 3 Relative potencies of drugs for [^{3}H]-bradykinin binding to canine TSMCs preincubated with LPS

Drug	IC_{50} (nM)	K_i (nM)
Agonists		
des-Arg^9-bradykinin	>100000	>100000
Bradykinin	34$\pm$9	13$\pm$4
Lys-Bradykinin	56$\pm$7	19$\pm$2
Antagonists		
[Leu^8, des-Arg^9]-bradykinin	>100000	>100000
[D-Arg^0,Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin	59$\pm$2	21$\pm$2
Hoe 140	18$\pm$4	6$\pm$1

Cultured TSMCs were preincubated in the presence of LPS (100 μg/ml) for 24 h. [^{3}H]-bradykinin (5 nM) binding was determined as described in Methods in the presence of increasing concentrations of drugs. Values are the mean$\pm$s.e.mean of three separate experiments.

Activation of MAPK requires Ras and Raf-1

Several lines of evidence have suggested that Ras plays an important role in a variety of cell functions mediated through sequential activation of Raf-1, MEK1/2, and MAPK (Blenis, 1993; Blumer & Johnson, 1994; Post & Brown, 1996; Panettieri, 1998). To elucidate whether the activation of Ras/Raf is required for MAPK phosphorylation induced by LPS and PDGF-BB, TSMCs were transfected with or without a dominant negative Ras (H-Ras-15A) or Raf (Raf-N4) and then treated with LPS or PDGF-BB. As shown in Figure 9, both LPS and PDGF-BB induced phosphrylation of p42/p44 MAPK in TSMCs. In addition, transfection with H-Ras-15A or Raf-N4 abolished p42/p44 MAPK phosphorylation induced by these two agents. There was no significant change in the phosphorylation of p42/p44 MAPK induced by LPS and PDGF-BB when TSMCs were transfected with empty vectors pZIP-NeoSV and pCGN, respectively. Parallel blot ran as controls that used antibody directed against the total p42 MAPK did not show any change (Figure 9). These results demonstrated that LPS shared a common mechanism with PDGF, activated Ras/Raf/MEK/MAPK pathway in TSMCs.

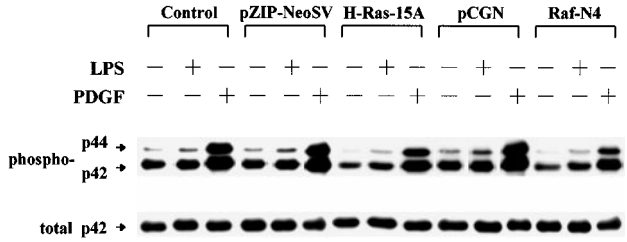

Figure 9 Requirement of Ras and Raf for LPS- and PDGF-BB-induced activation of p42/p44 MAPK in TSMCs. Cells were transfected with plasmids encoding pZIP-NeoSV, H-Ras-15A, pCGN, or Raf-N4, and then stimulated with LPS (100 μg ml^{-1}) or PDGF-BB (20 ng ml^{-1}) for 10 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with the anti-phospho-p42/p44 MAPK and total p42 MAPK (as a control) polyclonal antibody. Bands were visualized by an ECL method.

Discussion

Several lines of evidence suggest that LPS plays an important role in the regulation of cellular signalling cascades associated with airway inflammation in asthma (Platts-Mills & De Weck, 1989; Michel *et al*., 1996). In addition, LPS may contribute to enhance airway constrictor responsiveness *in vivo* (Michel *et al*., 1989, 1992; Hunt *et al*., 1994; Rylander *et al*., 1989; Sandstrom *et al*., 1992; Tamaoki *et al*., 1992). However, the mechanism(s) of LPS involved in airway hyperresponsiveness are largely unknown. The purpose of this study was to determine the mechanisms by which LPS enhances cellular responses to BK stimulation in canine cultured TSMCs. We demonstrate that (1) MAPK isoforms are activated by LPS *via* Ras/Raf/MEK/MAPK kinase cascade; (2) BK is a more effective stimulator of IPs accumulation and Ca^{2+} mobilization in canine TSMCs that had been exposed to LPS; (3) The increased sensitivity of $[Ca^{2+}]_i$ to BK in cells treated with LPS probably results directly from an enhanced accumulation of IPs. However, treatment of TSMCs with LPS did not enhance the carbachol-, ET-1-, and 5-HT-induced IPs response; (4) The site of action of LPS might be unique to the BK pathway and associated with BK receptor synthesis, which is, at least in part, mediated through the activation of p42/p44 MAPK, since PD98059 reversed these effects; and (5) The selective increase in the responsiveness of TSMCs to BK upon treatment of cells with LPS and PDGF-BB appears to result largely from an increase in the number of BK receptors. The mechanism underlying the enhancing effect of LPS most likely shares a common signalling pathway with PDGF-BB. The increase in expression of BK receptors probably contributed to the greater effectiveness of BK in inducing these responses.

LPS apparently contributed to the development of airway hyperresponsiveness and increased the airway resistance in response to bronchoconstricting stimuli (Michel *et al*., 1989; 1992; Hunt *et al*., 1994; Rylander *et al*., 1989; Sandstrom *et al*., 1992). Although the mechanisms of enhancement induced by LPS remain to be determined, there is evidence that one of mechanisms might be involved in the augmentation of transmembrane signalling. In our study, the potentiating effect of LPS was dependent on the incubation time and its concentrations. Short-term treatment with LPS is not sufficient to cause augmentation of IPs accumulation and Ca^{2+} mobilization induced by BK. However, long-term treatment (24 h) with LPS apparently enhances the BK-induced responses in these cells. Moreover, the concentration-effect curves of BK-induced IPs accumulation and Ca^{2+} mobilization were shifted to the left and the maximal responses were significantly enhanced in TSMCs treated with LPS as compared with the control cells. The EC_{50} values of BK-induced IPs accumulation and Ca^{2+} mobilization in TSMCs treated with LPS were close to those of non-treated cells. These results suggest that treatment with LPS did not change the affinity of BK receptors.

Although intracellular signals from the activation of specific tyrosine kinase-coupled growth factors have been well characterized, the mechanism by which LPS activates the MAPK cascade effectors in TSMCs is not completely understood. PD98059 is a synthetic and highly specific MEK1/2 inhibitor (Dudley *et al*., 1995). Because activation of components in the MAPK cascade originates from stimulation of cells by growth factors, it has been proposed that transmission of the signal along this pathway is required for the modulation of cellular functions. In current study, pretreatment with PD98059 attenuated LPS-induced activation of MAPK isoforms, potentiation of BK-induced responses, and expression of BK receptors in TSMCs, revealing that stimulation of MEK1/2 is required for LPS-induced responses in these cells, consistent with the involvement of MAPK in the up-regulation of BK receptor expression induced by LPS in vascular smooth muscle (Larrivee *et al*., 1998).

Furthermore, the enhancement of LPS on cellular responses might involve an up-regulation of cell surface receptor density or a change in the affinity. Several studies reported that LPS increases the expression of cell surface receptors or other proteins (Coin *et al*., 1996; Yasui *et al*., 1992; Marin-Castano *et al*., 1998) and thereby enhances cell signalling in response to various stimuli. In the present study, we found that treatment with LPS and PDGF-BB increased total [^{3}H]-BK binding sites in canine TSMCs. We speculate that LPS and PDGF-BB particularly increase the BK receptor number at the gene level, associated with protein synthesis, since TSMCs require to be exposed to LPS for several hours before any enhancing effect occurs. Furthermore, BK has a high affinity for B_2 and low affinity for B_1 receptors (Regoli *et al*., 1990). In contrast, des-Arg^9-BK has a high affinity for B_1, but low affinity for B_2 receptors (Regoli *et al*., 1990). In this study, the B_1 receptor-selective agonist, des-Arg^9-BK and antagonist, [Leu^8, des-Arg^9]-BK, triggered no significant displacement at concentrations up to 100 μM, thus excluding the presence of B_1 receptors in canine TSMCs. In contrast, the competitive inhibition of [^{3}H]-BK binding by the B_2 receptor-selective antagonists [D-Arg^0, Hyp^3, Thi^{5,8}, D-Phe^7]-BK and Hoe 140 and agonist BK showed a single population of BK binding sites with high affinities for B_2 receptors in canine TSMCs treated with LPS, consistent with the pharmacological properties of B_2 receptors as described for other tissues (Hess *et al*., 1994). Thus, the increase in BK receptors induced by LPS is primarily of B_2 receptors in TSMCs. This increase in BK receptor density is supported by the evidence that treatment with LPS did not affect carbachol-, ET-1-, and 5-HT-induced IPs accumulation. In addition, LPS might modulate BK receptor-coupled transduction mechanism such as G protein and thereby enhanced BK receptor-mediated signalling. However, LPS treatment did not modify AlF_4^--induced IPs accumulation (data not shown), which has been shown to directly stimulate G protein to generate IPs in TSMCs (Yang *et al*., 1994a), indicating that enhancement of LPS on BK-induced responses may be not located at the down stream of BK receptors. It is therefore conceivable that LPS specifically induces an increase in the number of BK receptors, which in turn potentiates BK-

induced IPs accumulation and Ca^{2+} mobilization in canine cultured TSMCs.

It has been well established that growth factors activate phosphorylation of protein kinases including tyrosine kinases, Ras, Raf-1, MEK and MAPK (Blenis, 1993; Blumer & Johnson, 1994; Post & Brown, 1996; Panettieri, 1998). The activation of protein kinases generally plays an important role in cell growth and gene expression (Blenis, 1993; Blumer & Johnson, 1994; Post & Brown, 1996; Panettieri, 1998). Several studies have depicted a picture that MAPK could be a convergence point for growth signals originating from tyrosine kinase receptors, G protein-coupled receptor, and cytokines (Blenis, 1993; Blumer & Johnson, 1994; Post & Brown, 1996; Panettieri, 1998). Several lines of evidence have suggested that Ras is activated by various stimuli for growth and differentiation (Satoh *et al*., 1992; Kerkhoff & Rapp, 1998) and that the activated Ras evoked the phosphorylation cascade of protein kinases including Raf-1, MEK1/2, and MAPK (Blenis, 1993; Blumer & Johnson, 1994; Post & Brown, 1996; Panettieri, 1998). Thus, it remains to be determined whether Ras plays a key role in the activation of p42/p44 MAPK induced by LPS in TSMCs. In this study, to elucidate whether Ras is required for LPS- and PDGF-BB-induced activation of MAPKs, TSMCs were transfected with H-Ras-15A that preferentially interacts with guanine nucleotide exchange factors and inhibits Ras functions (Chen *et al*., 1994; Cepko *et al*., 1984). We found that LPS- and PDGF-BB-induced p42/p44 MAPK activation were suppressed by transfection with the dominant negative mutant of Ras (H-Ras-15A) in TSMCs, as previously reported in other cell types (Chen *et al*., 1994; Abellatif *et al*., 1998). Several studies have also shown that activated Ras binds and activates Raf-1, resulting in the activation of MEK and MAPK (Blenis, 1993; Satoh *et al*., 1996, Brtva *et al*., 1995). Consistent with these reports, we demonstrated that in TSMCs, LPS- and PDGF-BB-induced MAPK activation are suppressed by transfection with a dominant negative mutant of Raf-1 (Raf-N4), suggesting that Raf-1 plays a key role in LPS- and PDGF-BB-induced activation of MEK/MAPK cascade in TSMCs.

In conclusion, our results demonstrate that treatment with LPS induces MAPK phosphorylation, BK receptor expression, and reinforces BK-induced IPs accumulation and Ca^{2+} mobilization in canine cultured TSMCs. Taken together, these studies suggest that LPS exerts a number of biological actions in the airways that may account for the induction of airway hyperresponsiveness in the asthma, through the activation of Ras/Raf/MEK/MAPK pathway in canine TSMCs.

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