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Tumour necrosis factor-α- and interleukin-1β-stimulated cell proliferation through activation of mitogen-activated protein kinase in canine tracheal smooth muscle cells

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1 The elevated levels of inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) have been found in the fluid of airways in symptomatic asthmatics. These cytokines have been considered as mitogens to stimulate cell proliferation in tracheal smooth muscle cells (TSMCs). We therefore investigated the effects of TNF-α and IL-1β on cell proliferation and activation of p42/p44 mitogen-activated protein kinase (MAPK) in these cells.

2 TNF-α and IL-1β induced [^{3}H]-thymidine incorporation in a time- and concentration-dependent manner. The maximal stimulation of [^{3}H]-thymidine incorporation induced by TNF-α and IL-1β was seen 12 h after incubation with these cytokines.

3 In response to TNF-α and IL-1β, p42/p44 MAPK was activated with a concentration-dependent manner in TSMCs. Pretreatment of TSMCs with pertussis toxin did not change DNA synthesis and phosphorylation of MAPK induced by TNF-α and IL-1β. These responses were attenuated by a tyrosine kinase inhibitor herbimycin, a phosphatidyl choline (PC)-phospholipase C (PLC) inhibitor D609, a phosphatidyl inositide (PI)-PLC inhibitor U73122, a protein kinase C inhibitor staurosporine, and removal of Ca^{2+} by addition of BAPTA/AM plus EGTA.

4 TNF-α- and IL-1β-induced [^{3}H]-thymidine incorporation and phosphorylation of p42/p44 MAPK was completely inhibited by PD98059 (an inhibitor of MEK1/2), indicating that activation of MEK1/2 was required for these responses.

5 These results suggest that the mitogenic effects of TNF-α and IL-1β were mediated through the activation of MEK1/2 and p42/p44 MAPK pathway. TNF-α- and IL-1β-mediated responses were modulated by PLC, Ca^{2+}, PKC, and tyrosine kinase associated with cell proliferation in TSMCs. *British Journal of Pharmacology* (2000) **130,** 891–899

Keywords: Cell proliferation; IL-1β; MAPK; protein kinase C; TNF-α; tyrosine kinase

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EC_{50}, concentration required for half-maximal stimulation; ECL, enhanced chemiluminescence; F-12, Ham's nutrient mixture F-12; FBS, foetal bovine serum; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK kinase; PC-PLC, phosphatidyl choline phospholipase C; PI-PLC, phosphatidyl inositide phospholipase C; PKC, protein kinase C; PTX, pertussis toxin; TNF-α, tumour necrosis factor-α; TSMCs, tracheal smooth muscle cells

Introduction

Asthma is a disease characterized by the presence of chronic inflammation, recruitment of pro-inflammatory cells into the airways, and airway remodelling. Remodelling of airway structure results in part from disruption of epithelium and smooth muscle hyperplasia (Dunnil, 1969; Heard & Hossain, 1973; Hossain, 1973) and hypertrophy (Ebina *et al*., 1993), which may exert as a major contributor of the airway hyperresponsiveness associated with asthma (James *et al*., 1989; Kuwano *et al*., 1993; Pare *et al*., 1991; Wiggs *et al*., 1992). The mechanisms underlying the hyperresponsiveness of the airways to a variety of stimuli are not known. One conceivable hypothesis is that pathological change at the level of the airway smooth muscle is implicated in asthma, since this tissue contributes the major contractile force for narrowing of the airways. There is emerging evidence that peptide growth factors proteases, low molecular weight bronchoconstrictors and inflammatory cytokines promote cell proliferation of tracheal smooth muscle cells (TSMCs). The increase in the mass of airway smooth muscle has been shown to correlate with airway hyperresponsiveness (Dunnil *et al*., 1969; Hossain, 1973; Armour *et al*., 1988; Sapienza *et al*., 1991; Hershenson *et al*., 1992).

There is increasing evidence that elevated levels of pro-inflammatory cytokines in the bronchoalveolar lavage fluid have been detected in allergic asthmatic patients (Broide *et al*., 1992; Mattoli *et al*., 1991; Watson *et al*., 1993; Kelley, 1990; Kips *et al*., 1992; Hamblin, 1991; Walker *et al*., 1992). These cytokines play an important role in the airway inflammatory responses in asthma. In inflamed airways like in asthma, denuded epithelium and an exposure of edematous basal membrane to airspace are seen (Laittinen *et al*., 1985). This indicates that, in asthma, these cytokines including TNF-α and IL-1β could therefore have a relatively easy access to TSMCs (Broide *et al*., 1992; Cembrzynska-Nowak *et al*., 1993; Gosset *et al*., 1991; Mattoli *et al*., 1991), which might have the potential to stimulate hypertrophic and/or hyperplastic

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changes in the airway wall smooth muscle. However, the precise role of cytokines implicated in these diseases remained to be established.

Several studies have demonstrated that TNF-α and IL-1β have stimulatory effects on cell proliferation in several cell types (Amrani *et al*., 1996; De *et al*., 1993; Stewart *et al*., 1995; Gehr *et al*., 1992; Delwel *et al*., 1992). However, little is known about the mechanisms of TNF-α and IL-1β that initiate the proliferation of TSMCs. It seems likely that, in analogy with the neointimal thickening process in atherosclerosis, TNF-α and IL-1β derived from inflammatory cells may play an important role in these processes. Moreover, many cytokines and growth factors have been shown to activate a signal transduction pathway that includes mitogen-activated protein kinase (MAPK) kinase and MAPK (Davids, 1993; Marshall, 1995). It has been well established that components of the MAPK pathway have been implicated as mediators of phosphorylation of intracellular substrates such as protein kinases and transcription factors (Karin, 1994) as well as regulators of cell growth and differentiation (Johnson *et al*., 1996). Among the most prominent tyrosine-phosphorylated proteins in TNF-α- and IL-1β-stimulated cells are the p42 and p44 isoforms of mitogen-activated protein kinase (MAPK) (Kyriakis & Avruch, 1996; LaPointe & Isenovic, 1999; Goetze *et al*., 1999). MAPKs are a family of serine/threonine protein kinases that participate in signalling pathways initiated by many extracellular stimuli. Once phosphorylated, these MAPKs then activate their specific substrates on serine and/or threonine residues to produce their effects on downstream targets. It has been demonstrated that these MAPKs are activated by TNF-α and IL-1β and by a wide variety of cellular stresses (Kyriakis & Avruch, 1996; LaPointe & Isenovic, 1999; Goetze *et al*., 1999). These findings further implicate the role of these MAPK pathways as important signalling mechanisms underlying the inflammatory process. Although TNF-α and IL-1β have been known to stimulate DNA synthesis, the cellular mechanisms for TNF-α and IL-1β-induced TSMC proliferation and activation of MAPKs, still need to be elucidated.

In this study, experiments were performed to characterize the relationship between cell proliferation and activation of p42/p44 MAPK induced by cytokines TNF-α and IL-1β using several pharmacologically relevant reagents. The results demonstrate that the mitogenic effects of TNF-α and IL-1β are mediated through the activation of MEK1/2 and p42/p44 MAPK pathway and associated with cell proliferation. Stimulatory effects of TNF-α and IL-1β are modulated by PLC, Ca^{2+}, PKC, and tyrosine kinase in cultured canine TSMCs.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium and foetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). [^{3}H]-Methyl thymidine, Hybond C membrane, and enhanced chemiluminescence (ECL) Western blotting detection system were from Amersham (Buckinghamshire, England, U.K.). BAPTA/AM, PD98059, herbimycin A, U73122, and D609 were from Calbiochem (San Diego, CA, U.S.A.). Recombinant human TNF-α and IL-1β were from R&D System (Minneapolis, MN, U.S.A.). PhosphoPlus p42/p44 MAPK antibody kits were from New England Biolabs (Beverly, MA, 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*., 1991; 1994a,b). 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}) DNase 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 and (10 ml per dish) 10 cm culture dish for [^{3}H]-thymidine incorporation and MAPK assay, 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.

[^{3}H]-thymidine incorporation

Proliferation studies were performed in confluent, growth-arrested TSMCs. Cells were plated onto 24-well culture plates and grown to confluence. TSMCs were growth-arrested by incubating the cells in serum-free DMEM/F-12 for 24 h. Confluent, growth-arrested cells were used because cells can be synchronized in G_0/G_1 phase of the cell cycle and at this baseline, minimally incorporate [^{3}H]-thymidine. After 24 h in serum-free media, the cells were stimulated with TNF-α or IL-1β. After 6 h of stimulation with TNF-α or IL-1β, TSMCs were labelled with 1 μCi ml^{-1} of [^{3}H]-thymidine for another 18 h in the continuous presence of TNF-α or IL-1β. When inhibitors were used, they were added 1 h prior to the application of TNF-α or IL-1β. The experiments were terminated by washing the cells with cold PBS, precipitation of acid-insoluble materials with 10% (w v^{-1}) TCA, and extraction of the DNA with 0.1 N NaOH. The precipitants were filtered through Whatman GF/B filters and washed three times with cold PBS using a cell harvester. The radioactivity was counted using a scintillation counter (Beckman LS5000TA, Fullerton, CA, U.S.A.).

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 TNF-α or IL-1β at 37°C for various times. When inhibitors were used, they were added 1 h prior to the

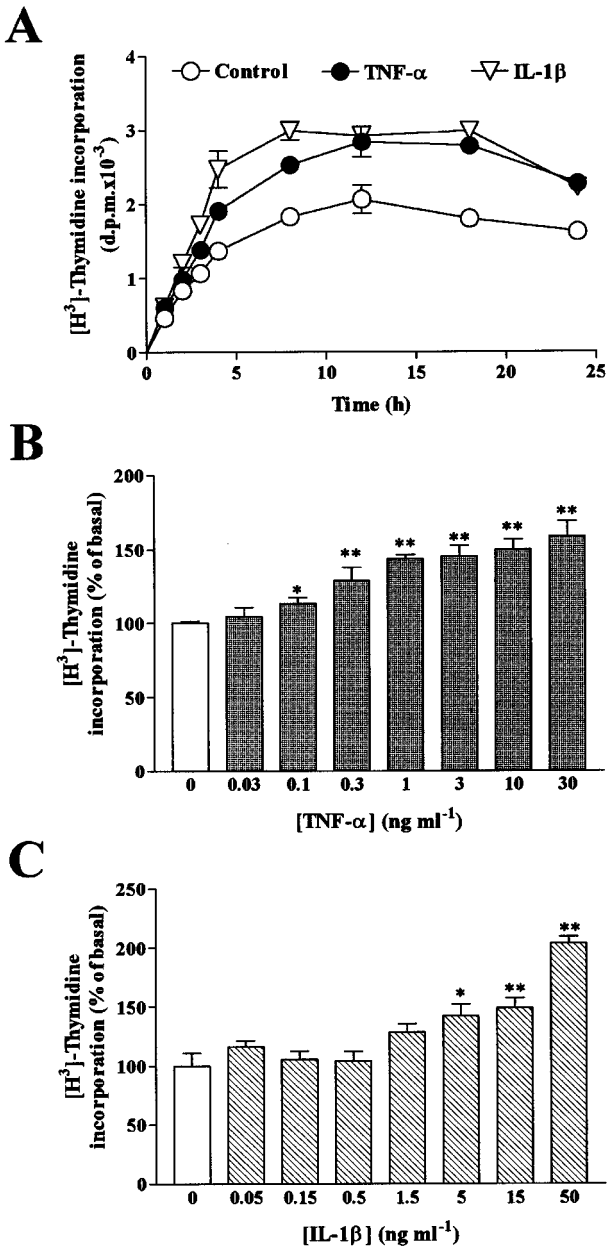

Figure 1 [^{3}H]-Thymidine incorporation induced by cytokines in TSMCs. For time course, after 24 h in serum-free medium, the cells were stimulated with vehicle (basal), 30 ng ml^{-1} TNF-α or 50 ng ml^{-1} IL-1β. The cells were labeled with 1 μCi ml^{-1} [^{3}H]-thymidine for the times indicated in the continuous presence of cytokines (A). For concentration dependence, the cells were stimulated with various concentrations of TNF-α (B) and IL-1β (C). After stimulation for 6 h, cells were labeled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 18 h in the presence of cytokines. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate. *$P<0.05$; **$P<0.01$, as compared with the basal level.

application of TNF-α or IL-1β. 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 (100 μg protein) were denatured and subjected to SDS–PAGE using a 10% (w v^{-1}) 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 for 1 h. The phosphorylation of p42/p44 MAPK isoforms was identified and quantified by Western blot analysis using Phospho-p42/44 MAPK antibody kits according to the recommendation of the manufacturer. Briefly, membranes were then incubated overnight at 4°C with the anti-phospho-MAPK polyclonal antibody used at 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 by Graph Pad Program (GraphPad, San Diego, CA, U.S.A.). Data were expressed as the mean$\pm$s.e.mean and analysed with a two-tailed Student's t-test at a $P<0.05$ level of significance.

Results

Effects of TNF-α and IL-1β on [^{3}H]-thymidine incorporation

In our previous studies, TSMCs appeared to be withdrawn from the cell cycle when cells were maintained in the serum-free medium (Yang *et al*., 1991). Under this condition, the kinetics of TNF-α and IL-1β on [^{3}H]-thymidine incorporation was observed in TSMCs. Figure 1A shows the influence of different incubation periods with TNF-α or IL-1β on the DNA synthesis in these cells. A significant [^{3}H]-thymidine incorporation was not seen until 4 h incubation and a maximal incorporation was obtained after incubation with TNF-α or IL-1β for 12 h. These results suggest that TNF-α and IL-1β induced [^{3}H]-thymidine incorporation in a time-dependent manner. Furthermore, the potency of TNF-α and IL-1β to stimulate DNA synthesis was evaluated in TSMCs. Incubation of TSMCs made quiescent by 24 h serum deprivation with increasing concentrations of TNF-α (Figure 1B) or IL-1β (Figure 1C) induced a marked elevation in DNA synthesis. This stimulation was concentration-dependent. Maximal stimulatory responses of TNF-α and IL-1β were obtained at concentrations of 30 and 50 ng ml^{-1}, respectively. The half-maximal effects (EC_{50}) of TNF-α and IL-1β on [^{3}H]-thymidine incorporation were 0.26$\pm$0.07 and 5$\pm$1 ng ml^{-1}, respectively.

Effects of TNF-α and IL-1β on the phosphorylation of MAPK isoforms

MAPKs, a group of components in the signal transduction pathway, have shown to be activated by several stimuli and associated with cell proliferation. Therefore, we determined whether TNF-α and IL-1β activated MAPKs in TSMCs. As shown in Figure 2, TSMCs were stimulated with various concentrations of TNF-α and IL-1β for 15 min, the phosphorylation of p42/p44 MAPK isoforms induced by TNF-α and IL-

1β was concentration-dependent. Densitometric analysis of the blots indicated that the maximal effects of TNF-α and IL-1β were achieved with 30 and 50 ng ml^{-1}, respectively (Figure 2). The half-maximal effects (EC_{50}) of TNF-α and IL-1β on the phosphorylation of p42/p44 MAPK isoforms were 0.31 ± 0.12 and 15 ± 5 ng ml^{-1}, respectively, which were closed to those of [^{3}H]-thymidine incorporation. It should be noticed that the EC_{50} values were estimated according to the range of concentrations used.

Effects of pertussis toxin and a tyrosine kinase inhibitor on DNA synthesis and p42/p44 MAPK phosphorylation

To examine the effects of pertussis toxin (PTX) and a tyrosine kinase inhibitor herbimycin A on DNA synthesis, TSMCs were pretreated with 100 ng ml^{-1} PTX for 24 h or 10 μM herbimycin A for 1 h, and then stimulated with TNF-α and IL-1β. As shown in Figure 3A, the TNF-α- and IL-1β-induced [^{3}H]-thymidine incorporation were significantly attenuated by pretreatment of these cells with herbimycin A ($P<0.01$, $n=3$), but not by PTX treatment ($P>0.01$, $n=3$). It should be noted that treatment with PTX significantly increased the basal level

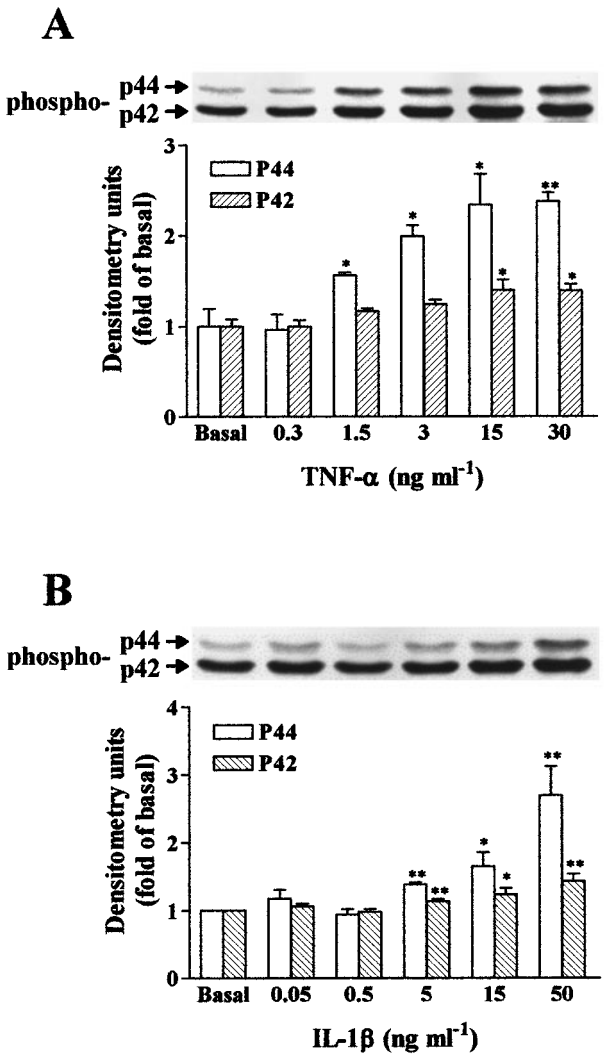

Figure 2 Concentration-dependence of TNF-α- and IL-1β-stimulated p42/p44 MAPK phosphorylation in TSMCs. The cells were grown to confluence, made quiescent by serum-deprivation for 24 h and incubated with various concentrations of TNF-α (A) and IL-1β (B) for 15 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with an anti-phospho-p42/p44 MAPK polyclonal antibody. Bands were visualized by an ECL method and quantified by a densitometer. Similar results were obtained in three independent experiments. Data are expressed as the mean$\pm$s.e.mean of three independent experiments. (Bar graph). *$P<0.05$; **$P<0.01$, as compared with the control cells exposed to respective cytokine.

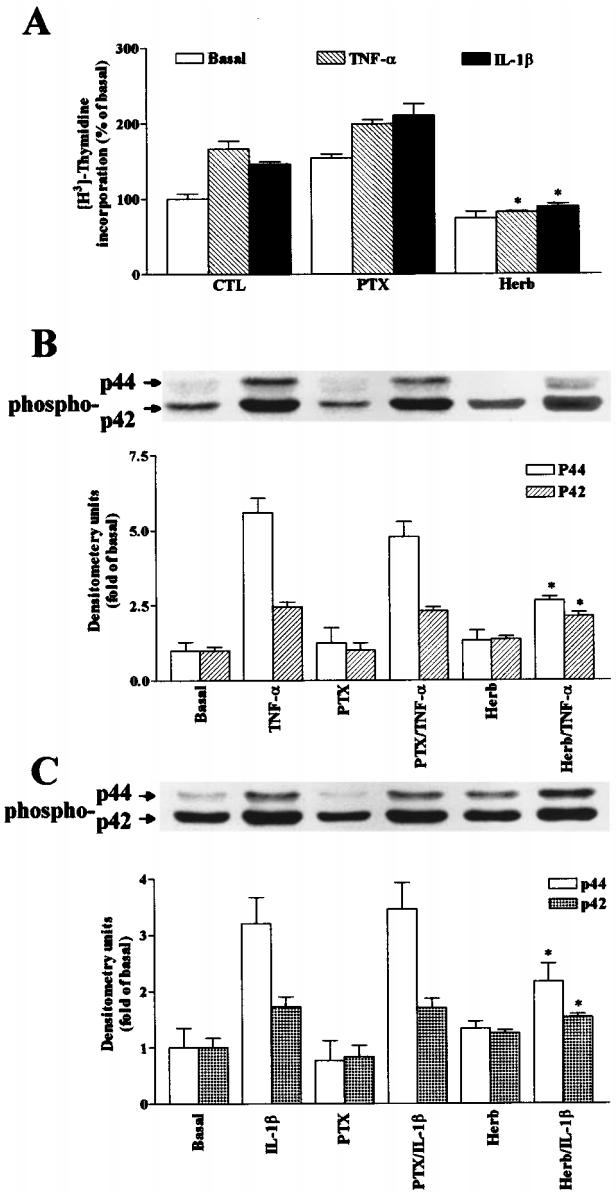

Figure 3 Involvement of G protein and tyrosine kinase in DNA synthesis and MAPK phosphorylation induced by cytokines in TSMCs. The cells were preincubated with pertussis toxin (PTX, 100 ng ml^{-1}, 24 h) or herbimycin A (Herb, 10 μM, 1 h), and then stimulated with vehicle, 30 ng ml^{-1} TNF-α or 50 ng ml^{-1} IL-1β. For DNA synthesis, after 6 h incubations, cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 18 h in the continuous presence of TNF-α or IL-1β. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate (A). For MAPK experiment, after treatment with these agents, the cells were stimulated with vehicle, 30 ng ml^{-1} TNF-α (B) or 50 ng ml^{-1} IL-1β (C) for 15 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. *$P<0.01$, as compared with the control cells exposed to respective cytokine.

of [^{3}H]-thymidine incorporation as compared with non-treated cells. The precise mechanism of action of PTX is not known.

To determine whether the effects of TNF-α and IL-1β on p42/44 MAPK activation were mediated through a PTX-sensitive G protein or activation of tyrosine kinase, TSMCs were treated with 100 ng ml^{-1} PTX for 24 h or 10 μM herbimycin A for 1 h, and then stimulated with 30 ng ml^{-1} TNF-α (Figure 3B) or 50 ng ml^{-1} IL-1β (Figure 3C) for 15 min. Pretreatment of these cells with herbimycin A inhibited the TNF-α- and IL-1β-induced p42/44 MAPK activation ($P<0.01$, $n=3$), but PTX had no effect on these responses. These results suggest that TNF-α- and IL-1β-induced phosphorylation of p42/p44 MAPK and DNA synthesis was mediated through activation of tyrosine kinase, but not through a PTX-sensitive G protein.

Effects of D609 and U73122 on DNA synthesis and p42/p44 MAPK phosphorylation

To investigate the effects of TNF-α and IL-1β on DNA synthesis were mediated through the activation of PI-PLC, [^{3}H]-thymisine incorporation was measured in TSMCs pretreated with U73122 before addition of TNF-α and IL-1β. As shown in Figure 4a, pretreatment of TSMCs with U73122 significantly attenuated the TNF-α- and IL-1β-induced [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$). Moreover, to characterize whether TNF-α- and IL-1β-induced phosphorylation of p42/p44 MAPK was mediated through the activation of PC-PLC or PI-PLC, TSMCs were pretreated with D609 or U73122 for 1 h and then stimulated with TNF-α (Figure 4B) or IL-1β (Figure 4C) for 15 min. Pretreatment of TSMCs with D609 or U73122 significantly inhibited the phosphorylation of p42/p44 MAPK induced by TNF-α and IL-1β ($P<0.01$, $n=3$). These results suggest that TNF-α and IL-1β-stimulated [^{3}H]-thymidine incorporation and p42/p44 MAPK activation was mediated through the activation of PC-PLC or PI-PLC in TSMCs.

Effect of PKC on DNA synthesis and p42/p44 MAPK phosphorylation

In order to determine whether PKC activation was involved in DNA synthesis in response to TNF-α and IL-1β, TSMCs were pretreated with a PKC inhibitor staurosporine (1 μM) for 1 h and then stimulated with TNF-α and IL-1β. As shown in Figure 5A, pretreatment of the cells with staurosporine attenuated [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$) induced by TNF-α and IL-1β. Moreover, to characterize whether TNF-α- and IL-1β-induced phosphorylation of p42/p44 MAPK was mediated through the activation of PKC, TSMCs were pretreated with a PKC inhibitor staurosporine (1 μM) for 1 h and then stimulated with TNF-α (Figure 5B) or IL-1β (Figure 5C) for 15 min. Pretreatment of TSMCs with staurosporine significantly inhibited the phosphorylation of p42/p44 MAPK induced by TNF-α and IL-1β ($P<0.01$, $n=3$). These results suggest that TNF-α- and IL-1β-stimulated [^{3}H]-thymidine incorporation and p42/p44 MAPK activation was mediated through the activation of PKC in TSMCs.

Effect of Ca^{2+} on DNA synthesis and p42/p44 MAPK phosphorylation

To elucidate whether Ca^{2+} was required for the [^{3}H]-thymidine incorporation, TSMCs were preincubated with 30 μM BAPTA/AM (a potent intracellular Ca^{2+} chelator) and 2 mM EGTA, and then stimulated with TNF-α and IL-1β. Results in Figure 6A demonstrate that pretreatment of these cells with BAPTA/AM reduced [^{3}H]-thymidine incorporation exposed to TNF-α and IL-1β ($P<0.01$, $n=3$). Furthermore, to determine whether Ca^{2+} was required for the activation of p42/p44 MAPK, Ca^{2+} was removed by addition of BAPTA/AM plus EGTA, and then TSMCs were stimulated with TNF-α (Figure 6B) and IL-1β (Figure 6C) for 15 min. The phosphorylation of p42/p44 MAPK induced by TNF-α and IL-1β was almost completely inhibited by removal of Ca^{2+} ($P<0.01$, $n=3$).

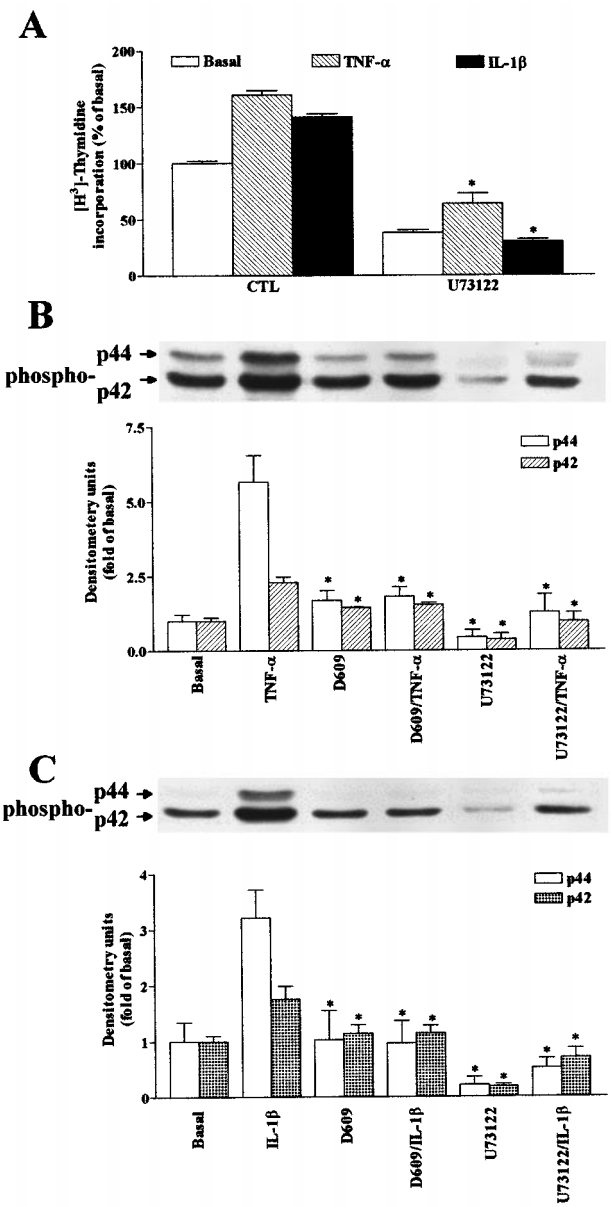

Figure 4 Effects of D609 and U73122 on DNA synthesis and MAPK phosphorylation induced by cytokines in TSMCs. The cells were preincubated with U73122 (10 μM, 1 h), and then stimulated with vehicle or cytokines. For DNA synthesis, after 6 h incubation, cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 18 h in the presence of 30 ng ml^{-1} TNF-α or 50 ng ml^{-1} IL-1β. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate (A). For MAPK experiment, after treatment with D609 (30 μM, 1 h) or U73122 (10 μM, 1 h), the cells were stimulated with vehicle, 30 ng ml^{-1} TNF-α (B) or 50 ng ml^{-1} IL-1β (C) for 15 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. *$P<0.01$, as compared with the control cells exposed to respective cytokine.

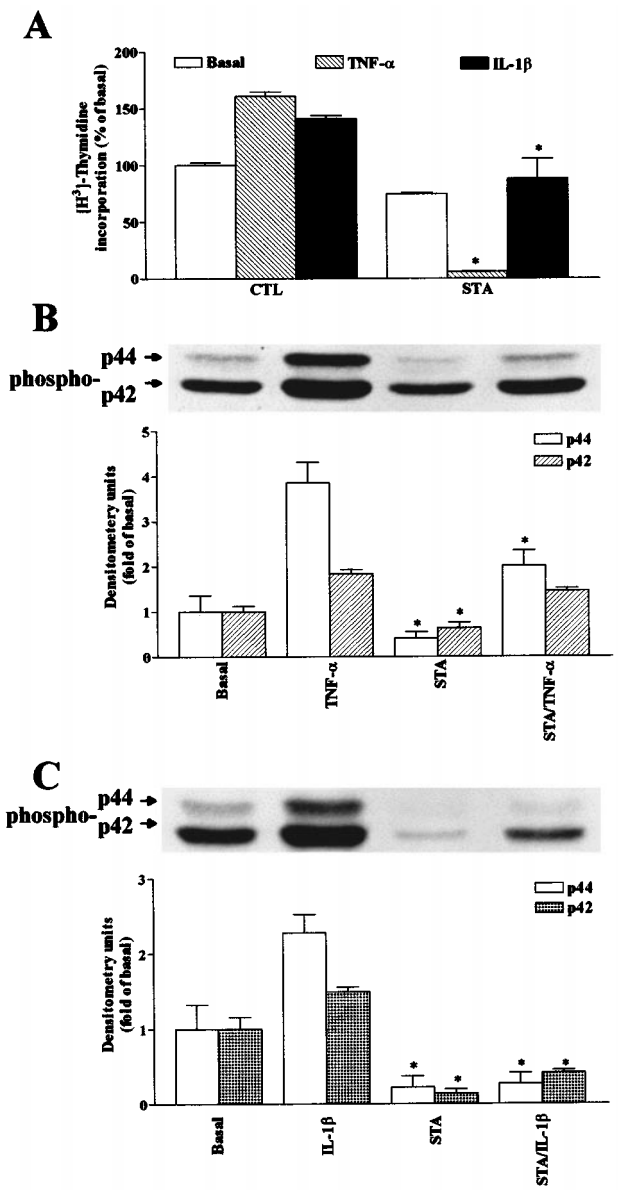

Figure 5 Effects of PKC on DNA synthesis and MAPK phosphorylation induced by cytokines in TSMCs. The cells were preincubated with staurosporine (STA, 1 μM, 1 h), and then stimulated with vehicle or cytokines. For DNA synthesis, after 6 h incubation, cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 18 h in the presence of 30 ng ml^{-1} TNF-α or 50 ng ml^{-1} IL-1β. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate (A). For MAPK experiment, after treatment with STA, the cells were stimulated with vehicle, 30 ng ml^{-1} TNF-α (B) or 50 ng ml^{-1} IL-1β (C) for 15 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. *$P<0.01$, as compared with the control cells exposed to respective cytokine.

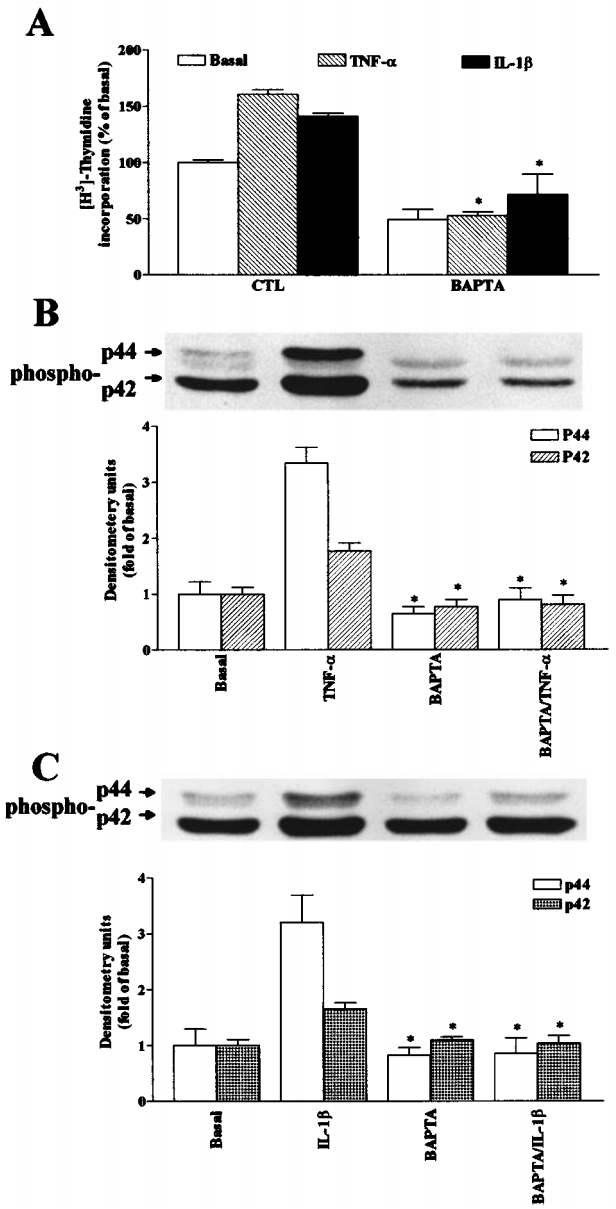

Figure 6 Effect of Ca^{2+} on DNA synthesis and MAPK phosphorylation induced by cytokines in TSMCs. The cells were preincubated with BAPTA/AM (10 μM) plus EGTA (2 mM) for 1 h, and then stimulated with vehicle or cytokines. For DNA synthesis, after 6 h incubation, cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 18 h in the continuous presence of 30 ng ml^{-1} TNF-α or 50 ng ml^{-1} IL-1β. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate (A). For MAPK experiment, after treatment with these agents, the cells were stimulated with vehicle, 30 ng ml^{-1} TNF-α (B) or 50 ng ml^{-1} IL-1β (C) for 15 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. *$P<0.01$, as compared with the control cells exposed to respective cytokine.

These results demonstrate that Ca^{2+} is required for the TNF-α and IL-1β-mediated [^{3}H]-thymidine incorporation and p42/p44 MAPK activation in TSMCs.

Effects of MAPK kinase inhibitors on DNA synthesis and p42/p44 MAPK phosphorylation

To ensure that the mitogenic effects of TNF-α and IL-1β are mediated through the activation of MAPK pathway, the effects of TNF-α and IL-1β on DNA synthesis were examined after treatment of TSMCs with 10 μM PD98059 (a synthetic MEK1/2 inhibitor) for 1 h. As shown in Figure 7A, treatment of TSMCs with PD98059 caused a significant inhibition of the TNF-α- and IL-1β-induced [^{3}H]-thymidine incorporation ($P<0.01$, $n=3$). In addition, the phosphorylation of p42/p44 MAPK induced by TNF-α (Figure 7B) or IL-1β (Figure 7C) was completely inhibited by PD98059, confirming that MEK1/2 is required for MAPK activation in these cells. These results

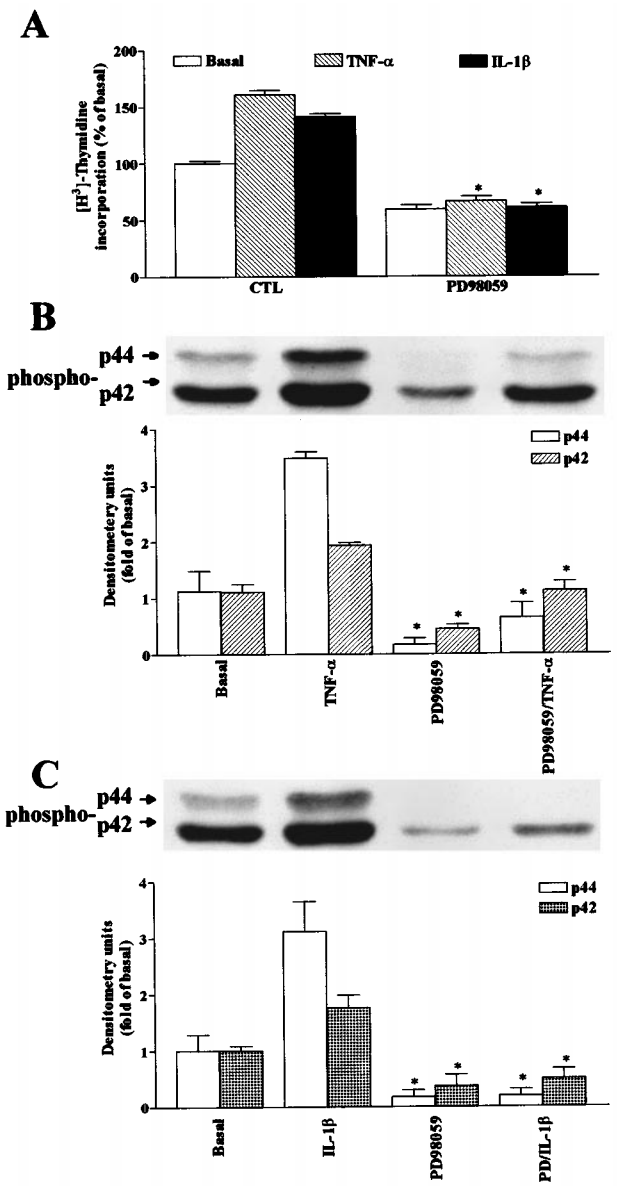

Figure 7 Effect of PD98059 on DNA synthesis and MAPK phosphorylation induced by cytokines in TSMCs. The cells were preincubated with PD98059 (30 μM, 1 h), and then stimulated with vehicle or cytokines. For DNA synthesis, after 6 h incubation, cells were labelled with 1 μCi ml^{-1} [^{3}H]-thymidine for another 18 h in the presence of 30 ng ml^{-1} TNF-α or 50 ng ml^{-1} IL-1β. The incorporation of [^{3}H]-thymidine was determined as described in Methods. Data are expressed as the mean$\pm$s.e.mean of three separate experiments determined in triplicate (A). For MAPK experiment, after treatment with PD98059, the cells were stimulated with vehicle, 30 ng ml^{-1} TNF-α (B) or 50 ng ml^{-1} IL-1β (C) for 15 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. *$P<0.01$, as compared with the control cells exposed to respective cytokine.

suggest that TNF-α- and IL-1β-induced phosphorylation of p42/p44 MAPK associated with cell proliferation was, at least in part, mediated through the activation of MEK1/2 in canine TSMCs.

Discussion

It is conceivable that several cytokines are implicated in the pathogenesis of many diseases associated with inflammation such as the acute respiratory distress syndrome, pulmonary fibrosis, and asthma. One of striking characteristics of asthma is airway hyperreactivity associated with an increase in airway smooth muscle mass caused by hypertrophy and hyperplasia (Pare *et al*., 1997; Ebina *et al*., 1993). Several studies demonstrated that TNF-α and IL-1β are potent mitogens to stimulate cell proliferation in various cell types including TSMCs (Amrani *et al*., 1996; De *et al*., 1993; Stewart *et al*., 1995; Gehr *et al*., 1992; Delwel *et al*., 1992), which contributes to the development of airway hyperreactivity. However, little is known about the intracellular signalling intermediaries that regulate cell proliferation associated with the activation of MAPK. In this study, we investigated the effects of TNF-α and IL-1β on phosphorylation of p42/p44 MAPK associated with cell proliferation in canine cultured TSMCs. TNF-α- and IL-1β-stimulated the activation of p42/p44 MAPK and [^{3}H]-thymidine incorporation were modulated by Ca^{2+}, PKC, PLC, and tyrosine kinase in these cells.

Although TNF-α and IL-1β bind to different membrane receptors, these cytokines may produce many similar cellular responses through common signalling pathways. The data obtained demonstrate that both TNF-α and IL-1β stimulate [^{3}H]-thymidine incorporation in a time- and concentration-dependent manner. These mitogenic effects may be mediated through the activation of p42/p44 MAPK in TSMCs. To determine whether TNF-α and IL-1β may interact with a G protein, the requirement of a G protein in DNA synthesis and activation of p42/p44 MAPK was evaluated in TSMCs pretreated with PTX for 24 h. Pretreatment of the cells with PTX has been shown to inhibit intrinsic GTPase activity of G_i protein by ADP-ribosylation of specific residues. Complete abrogation of PTX-sensitive G_i protein has been revealed by [^{32}P]-ADP-ribosylation of cell membranes prepared from TSMCs treated with PTX (Yang *et al*., 1994a). The results showed that TNF-α- and IL-1β-stimulated the activation of p42/p44 MAPK and [^{3}H]-thymidine incorporation was not affected by PTX treatment, excluding the involvement of a PTX-sensitive G protein in these processes.

Activation of p42/p44 MAPK is known to require both tyrosine and threonine phosphorylations by the dual specificity MEK1/2. Several lines of evidence indicate that complexity in the mechanisms for agonist stimulation of MAPK activities in several cell types including the possible involvement of tyrosine kinase upstream of MAPK kinase (Davis, 1993). MAPK isoforms are activated by various growth factors including PDGF (Kelleher *et al*., 1995), EGF (Pouyssegur & Seuwen, 1992), and TNF-α and IL-1β (Kyriakis & Avruch, 1996; LaPointe & Isenovic, 1999; Goetze *et al*., 1999). In this study, we have shown that stimulation of TSMCs with TNF-α and IL-1β resulted in activation of p42/p44 MAPK. These results are consistent with the findings that MAPK isoforms are activated by these cytokines in several cell types (Kyriakis & Avruch, 1996; LaPointe & Isenovic, 1999; Goetze *et al*., 1999). Furthermore, we investigated the implication of a tyrosine kinase in the MAPK cascade of TSMCs stimulated by TNF-α and IL-1β, using tyrosine kinase inhibitor herbimycin A. The results with the tyrosine kinase inhibitor showed that the TNF-α- and IL-1β-induced DNA synthesis and activation of p42/p44 MAPK was mediated through the activation of tyrosine kinase.

TNF-α and IL-1β have been shown to activate PC-PLC to generate DAG and enhances PKC activity in U937 cells (Schutze *et al*., 1991). PKC activation appears to be essential for late responses such as proliferation and differentiation (Nishizuka, 1992). PKC is a predominant component in the kinase cascade initiating by ligand attachment to both G protein coupled receptors and receptors containing intrinsic

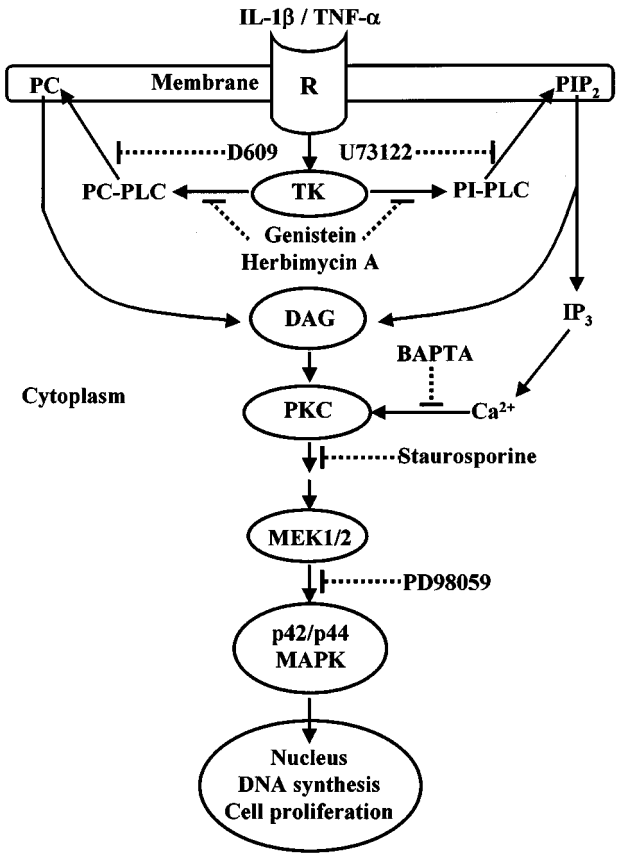

Figure 8 Schematic pathway for TNF-α and IL-1β signaling of cellular proliferation. Each solid line and arrow represents a step in an activating pathway. Each T-shaped and dashed line represents inactivation or inhibition. TNF-α and IL-1β bind to their receptors (R) and activate phosphatidylcholine-phospholipase C (PC-PLC) and phosphatidylinositide-phospholipase C (PI-PLC) through the phosphorylation of tyrosine kinase (TK) to induce protein kinase C (PKC) activation. Activation of PKC leads to sequential phosphorylation of MEK1/2 and p42/p44 MAPK that relays extracellular signalling into nuclei. TNF-α and IL-1β induce activation of the components of downstream p42/p44 MAPK and enhance DNA synthesis and cell proliferation in TSMCs.

tyrosine kinase activity. In this study, we further investigated the regulatory mechanisms which involved in TNF-α- and IL-1β-stimulated [^{3}H]-thymidine incorporation and activation of p42/p44 MAPK by PKC. These results demonstrated that pretreatment with a PKC inhibitor staurosporine attenuated the TNF-α- and IL-1β-stimulated [^{3}H]-thymidine incorporation and activation of p42/p44 MAPK, indicating that a component of the MAPK signal involves PKC-mediated activation of an intermediate kinase. This is likely to be either Raf-1 that has shown to be phosphorylated by PKC (Kolch *et al*., 1993) or possibly MEK1/2 that also believes to be activated in a PKC-dependent manner (Lange-Carter *et al*., 1993). Moreover, the mitogenic effects of TNF-α and IL-1β may be required the presence of Ca^{2+} in canine TSMCs. This hypothesis was supported by the results that removal of Ca^{2+} by BAPTA/EGTA significantly attenuated by p42/p44 MAPK activation and [^{3}H]-thymidine incorporation induced by these cytokines in canine cultured TSMCs. These results indicate an important role for Ca^{2+} in mediating the mitogenic effects of TNF-α and IL-1β.

Although mitogenic signals from the activation of specific tyrosine kinase-coupled growth factors have been well characterized, the mechanism by which the cytokines activate the components of MAPK pathway is not completely understood in TSMCs. PD98059, a synthetic and highly specific MEK1/2 inhibitor, has been shown to inhibit the activation of p42/p44 MAPK by several stimuli (Alessi *et al*., 1995; 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 induction of mitogenesis. In support of this hypothesis, inhibition of MEK1/2 by PD98059 has been associated with a decrease not only in PDGF-stimulated [^{3}H]-thymidine incorporation in 3T3 cells (Dudley *et al*., 1995), but also with an attenuation in nerve growth factor-induced differentiation in PC12 cells (Pang *et al*., 1995). In the current study, pretreatment with PD98059 attenuated the TNF-α- and IL-1β-induced activation of p42/p44 MAPK and DNA synthesis in TSMCs, revealing that stimulation of MEK1/2 is required for TNF-α and IL-1β-induced responses in these cells.

In conclusion, we report here that TNF-α and IL-1β appear to exert their mitogenic effects through the activation of MEK/MAPK pathway to enhance the DNA synthesis in canine cultured TSMCs. These stimulatory effects of cytokines are regulated by PKC, Ca^{2+}, PLC, and tyrosine kinase. Based on the observations from the literature and our findings, Figure 8 depicts a model for the role of p42/p44 MAPK activation associated with cell proliferation when TSMCs are exposed to TNF-α and IL-1β. These results also suggest that TNF-α and IL-1β may play an important role in the pathogenesis of asthma as well as structural changes seen in airway hyperreactivity.

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