Regulation of Toll-like receptor 4-induced proasthmatic changes in airway smooth muscle function by opposing actions of ERK1/2 and p38 MAPK signaling

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TOLL-LIKE RECEPTORS (TLRs) respond to a wide range of pathogen-associated molecular patterns that represent a host of microbial agents. TLR activation in cells comprising the innate immune system, including those coupled to stimulation of the adaptive immune response, has been implicated in initiating or facilitating pulmonary inflammation associated with various lung diseases such as acute respiratory distress syndrome, pulmonary tuberculosis, and chronic obstructive lung disease (7, 12, 37). Activation of TLRs has also been implicated in the pathobiology of asthma (6, 33, 38) in which respiratory infections may act to either exacerbate or prevent the clinical presentation of the disease (32, 36, 42). Recent studies that examined the potential role of TLRs on immune cells implicated in the pathogenesis of asthma, however, have yielded somewhat contradictory results. In this respect, whereas TLR4 activation with LPS has been reported to induce T helper lymphocyte type 2 (Th2) cytokine responses in a murine model of inhaled allergen sensitization (10), human monocyte-derived dendritic cells exposed to LPS were found to stimulate Th1 responses in cocultured naïve CD4+ T lymphocytes (1). Comparably, whereas the TLR2 and TLR9 synthetic agonists Pam3Cys and immunostimulatory sequence oligodeoxynucleotide (ISS-ODN), respectively, were shown to attenuate allergic airway inflammation and to promote Th1 responses in murine and primate models of allergic asthma (11, 23), TLR2 activation in human monocyte-derived dendritic cells was found to stimulate expression of Th2-type responses (1). While these seemingly contradictory results may be explained by possible species- and/or cell type-specific differences in the effects of activation of a given TLR, the latter studies support the general notion that different TLRs can exert opposing effects on allergic proinflammatory responses in the lungs.

The perturbed airway function in asthmatic individuals is characterized by enhanced airway constrictor responsiveness to bronchoconstrictor agents and impaired relaxation to β-adrenoceptor agonists (5, 16). In considering potential mechanisms responsible for induction of this airway asthmatic phenotype, it is relevant to note that beyond its inherent function as a regulator of airway tone, airway smooth muscle (ASM) has been found to directly respond to various proasthmatic stimuli (e.g., IgE immune complexes, certain viral respiratory pathogens, specific aeroallergens, etc.) that can elicit proasthmatic-like changes in its constrictor and relaxant responsiveness secondary to the autocrine actions of various cytokines released by the sensitized ASM itself (17, 19–21). Given this evidence, together with recent reports that ASM expresses certain TLRs that can alter its function (4, 35), the present study extended these previous findings by further systematically examining the potential role and mechanisms of action of...
TLR activation in regulating proinflammatory cytokine production and agonist-mediated constrictor and relaxation responsiveness in ASM. The results provide new evidence demonstrating that human ASM cells constitutively express mRNAs for TLR2 and TLR9 and that, in contrast to the minimal effect of TLR9 stimulation, activation of TLR4 with LPS potently elicits release of the pleiotropic proinflammatory cytokine IL-6 and evokes significant proaesthetic-like changes in rabbit ASM tissue constrictor and relaxant responsiveness. Furthermore, these effects are attributed to LPS-induced stimulation of the NF-κB transcriptional mechanism that is mediated by activation of the extracellular signal-regulated kinase (ERK)1/2 signaling pathway, whereas coactivation of the p38 MAPK pathway exerts an opposing (i.e., homeostatic) action by downregulating ERK1/2 signaling. Collectively, these observations elucidate the role and mechanism by which TLR4 activation in ASM regulates induction of proaesthetic changes in airway function. In this regard, the findings contribute to an improved understanding of the overall regulatory process that underlies the established association between endotoxin exposure and the induction of airways obstruction in asthma (6, 33, 38).

MATERIALS AND METHODS

Reagents

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. The human ASM cells were obtained from BioWhittaker (Walkersville, MD). Ham’s F-12 media were purchased from Mediatech (Herndon, VA), and 10% FBS was from Hyclone Laboratories (Logan, UT). The immunostimulatory oligodeoxynucleotide analog (ISS-ODN) 5'-TACGTGGAACGTTGATA (CpG B, K-type) was obtained from Integrated DNA Technologies (Corvallis, IA). The primary antibodies used for Western blotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the secondary antibodies and ECL reagents were obtained from Cell Signaling Technology (Beverly, MA). The nuclear protein extraction kit (Nucbuster) was obtained from Novagen (San Diego, CA), and the electrophoretic mobility shift assay (Gel-Shift) kit was from Panomics (Redwood City, CA). The MEK-ERK1/2 and p38 MAPK inhibitors, NF-κB essential modulator (NEMO) binding peptide, anisomycin, and MG132 were purchased from EMD Biosciences (Santa Cruz, CA), and Bay 11-7082 and SN50 were purchased from Alexis Biochemicals (San Diego, CA). The Pelikine Compact human IL-6 ELISA kit was obtained from Research Diagnostics (Flanders, NJ).

Animals

Twenty-two adult New Zealand White rabbits were used in this study, which was approved by the Biosafety and Animal Research Committee of the Joseph Stokes Research Institute at Children’s Hospital of Philadelphia. The animals had no signs of respiratory disease for several weeks before the study, and their care and use were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

Cell Culture and Exposure to TLR Agonists

The human ASM cells were grown in Ham’s F-12 media supplemented with 10% FBS and maintained throughout in a humidified incubator containing 5% CO₂ in air at 37°C. The experimental protocol involved growing the cells to 95% confluence in the above medium. Thereafter, in separate experiments, the cells were starved in unsupplemented Ham’s F-12 media for 24 h and then treated with different concentrations and for varying durations up to 48 h with either the TLR4 agonist, LPS, purified from Escherichia coli 055:B5 (Sigma), or the TLR9 agonist, ISS-ODN. At indicated times after the administration of each TLR agonist, aliquots of the cell culture medium were collected for assay of IL-6 protein concentration using an ELISA kit.

Detection of TLR2, TLR4, and TLR9 mRNA transcripts

Total RNA was extracted from the cultured ASM cells using the TRizol method (Invitrogen, San Diego, CA). RT-PCR was conducted to isolate cDNAs of TLR2, TLR4, and TLR9 using the following primer sets purchased from Integrated DNA Technologies: for TLR2, 5'-GTGACGTGTCGGAGGTTCGCT-3' (forward) and 5'-GTTCCACTATTTCCACCTCGAGG-3' (reverse); for TLR4, 5'-AGGAAGCCTGAGGTCTGGATT-3' (forward) and 5'-TCTTTAAATGCACCTGTTGGT-3' (reverse); and for TLR9, 5'-GCTGTCCTCCTCGTGTCGACG-3' (forward) and 5'-GGATAAGTACCTGCCGTTGA-3' (reverse). The reaction volume was 20 μl, and cycling conditions used were 35 cycles of 30-s denaturation at 95°C, followed by 30-s annealing at 60°C and elongation at 72°C for 30 s. Ex-Tag (Takara Biotechnology, Shiga, Japan) was used as DNA polymerase. The PCR products were subsequently sequenced to confirm that they represent the reported transcripts for the different TLRs, as accessed from the GenBank database.

Immunoblot Analysis of MAPK Activation

Phosphorylated and total ERK1/2, p38 MAPK, and JNK protein levels were assessed by Western blot analysis of lysates isolated from ASM cells before and after treatment for 5, 15, 30, and 60 min with either LPS (0.1 μg/ml) or ISS-ODN (2 μg/ml), as previously described (17). After protein extraction and the addition of gel loading buffer, the extracts were loaded onto a 10% SDS-PAGE gel for immunoblotting after transfer to a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk for 1 h and incubated overnight with monoclonal mouse anti-human primary antibodies including phosphoERK(Thr183/Tyr185)-JNK and JNK1. The kinase levels were determined with a 1-h incubation with a 1:1,000 dilution of horseradish peroxidase (HRP)-conjugated rabbit anti-mouse secondary antibody, followed by exposure to autoradiography film. The protein band intensities were quantified by densitometry.

EMSA Analysis of NF-κB Binding

After serum deprivation for 24 h, confluent cultures of ASM cells were treated with LPS in the absence and presence of pretreatment for 30 min with specific MAPK or NF-κB pathway inhibitors, as indicated. Thereafter, the cells were harvested and nuclear extracts were prepared using a protein extraction kit (Novagen). The nuclear extracts (5 μg) were then incubated with a biotin-tagged oligodeoxynucleotide probe (5'-AGTTGAGGGGACTTTCCCAGGC) containing the DNA consensus binding site for NF-κB and, for supershift assays, a rabbit polyclonal anti-p65 antibody (Santa Cruz Biotechnology) was added to the nuclear extracts, according to the protocol described in the EMSA kit from Panomics. The extracts were electrophoresed using a 5% polyacrylamide gel at 4°C and transferred to Biodyne B nylon membrane (Pall, East Hills, NY). The blots were visualized by ECL.

Coimmunoprecipitation Studies

Confluent cultures of serum-deprived untreated and LPS-treated ASM cells were prepared for nuclear extraction as above and, following centrifugation, supernatants containing the cytosolic fraction were
also isolated according to the protocol described in the protein extraction kit. A polyclonal anti-p65 antibody was added to the soluble extracts and, after overnight incubation, immune complexes were precipitated with protein A-Sepharose beads. The pellets were washed \(3 \times\) with PBS buffer and then loaded onto a 10% SDS-PAGE gel for immunoblotting after transfer to a PVDF membrane. The nuclear and cytosolic samples were probed with monoclonal mouse anti-human antibodies directed at phosphorylated ERK1/2 and p65. Expression of these proteins was detected by ECL after incubation with HRP-conjugated rabbit anti-mouse secondary antibodies, followed by exposure to autoradiography film.

**Preparation and Treatment of Rabbit ASM Tissues**

After initial sedation and subsequent general anesthesia with intramuscular injections of xylazine (10 mg/kg) and ketamine (50 mg/kg), respectively, rabbits were killed with an intravenously administered overdose of pentobarbital (125 mg/kg). As described previously (21), the tracheae were removed via open thoracotomy, the loose connective tissue and epithelium were scraped and removed, and the tracheae were divided into eight ring segments, each of which was 6–8 mm in length. The airway segments were then placed in modified Krebs-Ringer solution containing indomethacin (10 \(\mu\)M), and each alternate ring was incubated for 24 h at room temperature in the presence of either vehicle alone (control) or varying concentrations of LPS both in the absence and presence of either the MEK-ERK1/2 inhibitors U0126 (5 \(\mu\)M) or PD-98059 (10 \(\mu\)M), the p38 MAPK inhibitors SB-203580 (10 \(\mu\)M) or SB203580 (10 \(\mu\)M), or the JNK inhibitor SP-600125 (1 \(\mu\)M). In parallel experiments, ASM segments were exposed to LPS in the absence and presence of pretreatment with either the IkBo inhibitor Bay 11-7082 (20 \(\mu\)M) or the proteasome inhibitor MG132 (50 \(\mu\)M). Finally, in separate studies, 1 h before incubation in control or LPS-containing medium, ASM tissues were treated with the potent activator of p38 MAPK, anisomycin, using a concentration (i.e., 50 ng/ml) that is significantly less than that required to prevent the known protein translational inhibitory effect of anisomycin (24, 31). All the tissues studied were continuously aerated with a gas mixture containing 95% \(O_2\) and 5% \(CO_2\) throughout the incubation phase.

**Pharmacodynamic Studies of ASM Constrictor and Relaxant Responsiveness**

After being incubated, the tissues were placed in organ baths containing modified Krebs-Ringer solution aerated with 5% \(CO_2\) in oxygen (pH of 7.35–7.40), and the tissues were attached to force transducers from which isometric tension was continuously displayed on a multichannel recorder, as previously described (21). Cholinergic contractility was then assessed in the ASM segments by cumulative administration of ACh in final bath concentrations ranging from \(10^{-9}\) to \(10^{-3}\) M. Thereafter, the tissues were repeatedly rinsed with fresh buffer and, subsequently, relaxation dose-response curves to isoproterenol (\(10^{-9}\) to \(10^{-4}\) M) were generated after the tissues were half-maximally contracted with their respective ED\(_{50}\) doses of ACh. The initial constrictor dose-response curves to ACh were analyzed in terms of each tissue’s maximal isometric contractile force (T\(_{max}\)) to the agonist. The subsequent relaxation responses to isoproterenol were analyzed in terms of % maximal relaxation (R\(_{max}\)) from the initial level of active cholinergic contraction.

**Statistical Analysis**

The results are expressed as means ± SE. Comparisons between groups were made using the Student’s \(t\)-test (two-tailed) or ANOVA with Tukey’s post-test analysis, where appropriate. \(P < 0.05\) was considered statistically significant. Statistical analyses were conducted using the Prism computer program by GraphPad Software (San Diego, CA).

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**RESULTS**

**TLR Expression and Action in Cultured Human ASM Cells**

Because TLR2, TLR4, and TLR9 have been associated with either the induction or inhibition of proinflammatory allergic pulmonary responses (1–3, 12, 13), we examined whether transcripts for these TLRs are expressed in the cultured human ASM cells. RNA was extracted from confluent ASM cells and, relative to constitutively expressed \(\beta\)-actin transcripts, PCR reactions using primers for these TLRs demonstrated a strong band representing the TLR4 transcripts, whereas TLR9 transcripts were faintly expressed, and no PCR products from TLR2 transcripts were detected (Fig. 1A). The TLR4 and TLR9 transcripts were of expected sizes of 441 and 503 bp, respectively, and both these PCR products were subsequently sequenced to confirm their identity with the reported transcripts of TLR4 and TLR9 accessed from the GenBank database.

In light of these results, and given that the pleiotropic cytokine, IL-6, has been importantly implicated in mediating proinflammatory events that characterize the transition from innate to acquired immunity in experimental allergic asthma (8, 27), we next examined whether activation of TLR4 and TLR9 modulates release of IL-6 from the cultured ASM cells. Treatment of the ASM cells with the TLR4 agonist, LPS,
elicited time- and dose-dependent increases in IL-6 accumulation in the cell culture medium (Fig. 1B). For each administered dose of LPS, the peak increase in IL-6 accumulation was detected at 24 h, and maximal IL-6 release was elicited with an LPS dose of 1 μg/ml. Contrasting this response to LPS, cells treated with the TLR9 agonist, ISS-ODN, exhibited minimal induced IL-6 release, including with the highest administered dose of the agonist (i.e., 2.0 μg/ml), and the levels of IL-6 accumulation were not significantly different from those detected in control (untreated) cells, which averaged 1.8 ng/ml over 48 h.

Role of MAPK Activation in TLR-Stimulated Human ASM Cells

Previous studies have implicated activation of MAPKs and NF-κB in mediating the effects of specific TLRs in various cell types (40). In light of this evidence, we initially examined the role of MAPK activation by assessing TLR4 and TLR9 agonist-induced expression of the phosphorylated forms of ERK1/2, p38 MAPK, and JNK. Western blots were performed using antibodies specific for these phosphorylated kinases as well as antibodies directed against total ERK, p38 MAPK, and JNK proteins. As depicted in Fig. 2A, LPS elicited enhanced expression of phosphorylated ERK1/2, which peaked at 15 min and was subsequently distinctly reduced at 60 min. ISS-ODN also induced ERK1/2 phosphorylation; however, this effect was notably less pronounced than that elicited by LPS. By comparison, increased expression of phosphorylated p38 MAPK was detected as early as 5 min in both the LPS- and ISS-ODN-treated ASM cells, whereas phosphorylated JNK expression was affected little by either of the TLR agonists. Of note, neither the total protein levels of ERK1/2, p38, nor JNK were systematically affected by treating the cells with LPS or ISS-ODN (data not shown). Accordingly, based on densitometric analysis of the results obtained in three experiments, as indicated in Fig. 2A, bottom, maximal levels of phosphorylated ERK1/2, p38 MAPK, and JNK expression detected in LPS-exposed ASM cells averaged 4.94-, 1.52-, and 1.11-fold, respectively, above the basal (pretreatment) levels and, correspondingly, in ISS-ODN-treated cells, maximal expression of these MAPKs averaged 2.11-, 1.61-, and 1.07-fold, respectively, above the basal determinations.

In extending these observations to assess the potential role of MAPK activation in regulating LPS-induced IL-6 release, we next examined the effects of selective inhibitors of the MEK-ERK1/2, p38 MAPK, and JNK pathways on IL-6 release from LPS-stimulated ASM cells. As shown in Fig. 2B, relative to the mean ± SE level of IL-6 detected in the culture medium of control (untreated) cells, which amounted to 1.7 ± 0.9 ng/ml, the levels of IL-6 were significantly increased by a mean of 4.9 ± 0.9-fold above control in the medium of cells exposed for 24 h to a submaximal dose of LPS (0.1 μg/ml). Pretreatment of LPS-exposed cells with either the JNK inhibitor SP-600125 (1 μM) or the p38 MAPK inhibitor SB-202190 (10 μM) had no significant effect on LPS-induced IL-6 accumulation. However, the LPS-induced release of IL-6 was inhibited in cells pretreated with the MEK-ERK1/2 inhibitor U0126 (5 μM), yielding levels of IL-6 accumulation that were not significantly different from those detected in control cells.

Role of NF-κB Activation in LPS-Stimulated ASM Cells

Together with MAPK activation, stimulation of TLRs in various cell types has also been associated with activation of NF-κB. Characteristically, the induced activation of NF-κB requires phosphorylation of NF-κB-bound inhibitory IκB proteins by IKK, a molecular complex composed of the regulatory subunit, NEMO (also called IKKγ), and the kinase subunits IκKα and IκKβ. This leads to removal of IκB by proteasome-mediated degradation, thereby enabling liberated NF-κB to translocate to the nucleus and promote proinflammatory gene transcription (43). To evaluate the role of NF-κB activation in
mediating LPS-stimulated IL-6 release from the ASM cells, we initially examined the effects of specific inhibitors of different components of the NF-κB signaling mechanism on LPS-induced NF-κB activity. The latter was assessed by EMSA analysis of NF-κB binding to its consensus sequence contained in a biotin-labeled DNA probe. As shown in Fig. 3, relative to basal NF-κB/DNA binding activity detected in control (unstimulated) cells, LPS-stimulated ASM cells exhibited distinctly enhanced NF-κB binding. This augmented NF-κB binding activity was inhibited in LPS-stimulated cells pretreated with either an inhibitory NEMO binding peptide (NEMO-BP); BAY 11-7082, an inhibitor of IκBα phosphorylation; MG132, a proteasome inhibitor; or SN50, an inhibitor of NF-κB translocation to the nucleus. Thus these data provided evidence that LPS-induced NF-κB activation in ASM cells is mediated by stimulation of the classic IKK/IκBα-coupled NF-κB activating mechanism. In relation to these studies, it should be noted that the specificity of NF-κB binding activity was demonstrated by the absence of a detectable EMSA band when administering a 100-fold excess of unlabeled (“cold”) DNA oligomer to compete for NF-κB binding with the labeled DNA probe (data not shown).

In view of these observations, we next examined the effect of inhibition of NF-κB signaling on LPS-induced IL-6 release from the ASM cells. As demonstrated in Fig. 4, the induced release of IL-6 from cells exposed for 24 h to LPS (0.1 μg/ml) was completely abrogated when the LPS-stimulated cells were pretreated with the IκBα inhibitor BAY 11-7082 (10 μM). Qualitatively similar effects were also obtained when using a higher concentration of administered LPS (i.e., 1 μg/ml) and when pretreating the cells with other NF-κB pathway inhibitors, including NEMO-BP (100 μM) or MG132 (100 μM) (data not shown).

**Fig. 3.** EMSA analysis of nuclear protein extracts showing NF-κB binding activity in human ASM cells under control conditions (lane 1) and at 20 min after treatment with LPS (0.1 μg/ml) in the absence and presence of pretreatment of the cells for 30 min with various inhibitors of NF-κB signaling. LPS-induced NF-κB binding activity (lane 2) is inhibited by pretreatment either with NF-κB essential modulator binding peptide (NEMO-BP; 100 μM, lane 3), BAY 11-7082 (10 μM, lane 4), MG132 (100 μM, lane 5), or SN50 (20 μM, lane 6). The EMSA is representative of 3 separate experiments.

Since inhibition of either ERK1/2 or NF-κB signaling was found to be associated with ablation of LPS-induced IL-6 release (Figs. 2B and 4, respectively), we next investigated the potential causal interplay between these signaling pathways in LPS-stimulated cells. In addressing this issue, NF-κB binding activity was examined by EMSA analysis of nuclear extracts from control (unstimulated) and LPS-stimulated ASM cells in the absence and presence of pretreatment with either an ERK1/2 or p38 MAPK inhibitor. As depicted in Fig. 5A, relative to the basal activity detected in control cells (Fig. 5A, lane 1), NF-κB binding activity was distinctly enhanced in cells treated for 30 min with LPS (Fig. 5A, lane 2) and, as shown by the EMSA supershift (Fig. 5A, lane 5), this augmented NF-κB binding involved the p65 subunit of NF-κB. The enhanced NF-κB binding activity was largely ablated in LPS-exposed cells pretreated with the MEK-ERK1/2 inhibitor U0126 (Fig. 5A, lane 3). Contrasting the latter observation, pretreatment of cells with the p38 MAPK inhibitor SB-202140 had no inhibitory effect on LPS-induced stimulation of NF-κB binding activity (Fig. 5A, lane 4). Indeed, densitometric analysis of the results obtained in three experiments demonstrated that the intensities of the LPS-induced NF-κB/DNA binding signals detected in cells pretreated with the p38 MAPK inhibitor were increased by an average of 1.32-fold above that obtained in cells exposed to LPS alone.

In light of the above results, to further substantiate the suspected role of activated ERK1/2 in mediating LPS-induced NF-κB activity, we next assessed whether stimulation with LPS evokes coupling of phosphorylated ERK1/2 with NF-κB. In this context, it is relevant to note that phosphorylated ERK1/2 is known to exert its wide range of biological actions through phosphorylation of cytoplasmic and/or nuclear substrates. Accordingly, cytosolic and nuclear extracts were prepared from control (untreated) cells and cells pretreated for 30
Relative to control tissues, ASM segments treated for 24 h with a maximally effective dose of LPS (1 μg/ml) exhibited increased constrictor responses to exogenously administered Ach (Fig. 6). Accordingly, relative to the mean ± SE maximal constrictor response (Tmax) obtained in control ASM (i.e., 91.3 ± 9.1 g/g ASM wt), the mean Tmax response was significantly increased at 104.6 ± 7.9 g/g ASM wt in LPS-exposed tissues (P < 0.05). This enhanced constrictor responsiveness to Ach was completely abrogated in LPS-exposed ASM pretreated with the MEK-ERK1/2 inhibitor U0126 (5 μM), providing Tmax values similar to those generated in control ASM segments. Contrasting the latter effect of ERK1/2 inhibition, LPS-exposed tissues pretreated with the p38 MAPK inhibitor SB-202190 (10 μM) exhibited a trend further toward enhanced constrictor responsiveness to Ach, yielding a mean Tmax value of 111.2 ± 8.8 g/g ASM wt. Moreover, in additional experiments, we found that, with ERK1/2 inhibition, the increased constrictor responsiveness to Ach was also completely abrogated in LPS-exposed tissues pretreated with either the IkBα inhibitor BAY 11-7082 (10 μM) or the proteasome inhibitor MG132 (100 μM) (data not shown).

Under the same treatment conditions, during subsequent sustained high-maximal contraction with Ach, administration of the β-adrenoceptor agonist isoproterenol produced cumulative dose-dependent relaxation of the precontracted ASM segments. As shown in Fig. 7, relative to control ASM, the relaxation responses to isoproterenol were significantly attenuated in the LPS-exposed ASM in which the mean Rmax responses amounted to 40.3 ± 3.0% vs. 59.1 ± 3.8% obtained in the control tissues (P < 0.01). This impaired relaxation responsiveness was prevented in LPS-exposed tissues pretreated with U0126, whereas LPS-exposed ASM segments pretreated with SB-202190 exhibited further significantly impaired (P < 0.05) relaxation responses to isoproterenol, yielding a mean Rmax value of 34.8 ± 4.7%. Moreover, in extended

Effects of LPS on Rabbit ASM Tissue Responsiveness

Roles of MAPKs and NF-κB activation. To further assess the effects and mechanism of action of TLR4 activation on ASM function, we next compared the agonist-mediated constrictor and relaxation responses in control and LPS-exposed isolated rabbit ASM segments, both in the absence and presence of pretreatment with the above MAPK and NF-κB inhibitors.
Fig. 7. Modulatory effects of MAPK inhibitors on LPS-induced changes in relaxation responsiveness to isoproterenol in rabbit ASM tissues. Isolated rabbit ASM tissues were exposed for 24 h to vehicle alone (control, ○) or to LPS (1 μg/ml) in the absence (●) or presence of pretreatment for 30 min either with U0126 (5 μM, □) or with SB-202190 (10 μM, △). Relaxation responses to cumulatively administered isoproterenol were generated after initial sustained half-maximal constriction of the tissues with ACh. Relative to control tissues, maximal relaxation (Rmax) responses to isoproterenol were significantly impaired in LPS-exposed tissues pretreated with SB-202190, whereas the relaxation responses were unaltered in LPS-exposed tissues pretreated with U0126. Data represent means ± SE values from 4 separate experiments.

Studies found that, as with the above MAPK inhibitors, in separate experiments we found qualitatively comparable opposing effects of ERK1/2 and p38 inhibition on ASM responsiveness in LPS-exposed tissues pretreated with other selective MAPK inhibitors including the ERK1/2 inhibitor PD-98059 and the p38 MAPK inhibitor SB-203580 (data not shown). Additionally, contrasting with their effects in LPS-exposed tissues, neither of these inhibitors produced a significant change in constrictor or relaxation responsiveness when administered to tissues not exposed to LPS (data not shown).

Regulatory Effects of p38 MAPK on LPS-Induced NF-κB Activation and Changes in ASM Responsiveness

The above results suggested that, contrary to the proasthmatic-like effects of ERK1/2-mediated NF-κB activation in ASM responsiveness, coactivation of p38 MAPK exerted an opposing protective (homeostatic) action on LPS-induced changes in ASM responsiveness. To further substantiate this opposing action of p38 MAPK, we assessed the effects of selectively induced p38 MAPK activation on both LPS-induced stimulation of NF-κB/DNA binding activity and changes in ASM responsiveness. Accordingly, EMSA analysis of NF-κB binding was compared in control and LPS-exposed ASM cells in the presence and absence of pretreatment with anisomycin, a potent activator of p38 MAPK. As depicted by a representative experiment in Fig. 8, relative to unstimulated (control) cells, NF-κB activity was strongly induced in LPS-stimulated cells and, as described above, this effect was largely abrogated in LPS-exposed cells pretreated with the MEK-ERK1/2 inhibitor U0126. Pretreatment with anisomycin (50 ng/ml) alone had little effect on basal NF-κB activity; however, the induced enhanced NF-κB activity exhibited by LPS-stimulated ASM cells was completely ablated when LPS-exposed cells were pretreated with anisomycin. Moreover, this inhibitory effect of anisomycin was unaltered in LPS-exposed cells that were concomitantly pretreated with the JNK inhibitor SP-600125 (1 μM), implying that the action of anisomycin was attributed to its stimulation of p38 MAPK, whereas its potential to coactivate JNK had no appreciable effect. Thus, together, these observations support the concept that activation of p38 MAPK in LPS-exposed ASM serves, at least in part, to limit the magnitude of ERK1/2-mediated activation of NF-κB.

In related experiments, next examined whether the negative regulatory action of p38 MAPK activation on NF-κB binding activity is coupled to modulatory effects of p38 MAPK activation on LPS-induced ERK1/2 phosphorylation and associated changes in ASM responsiveness. As illustrated by a representative experiment in Fig. 9A, the induced enhanced expression of phosphorylated ERK1/2 in LPS-stimulated human ASM cells was largely abrogated when the LPS-exposed cells were pretreated with anisomycin, whereas the latter had no effect on total ERK2 protein expression. Comparably, pretreatment with anisomycin (50 ng/ml) also inhibited the proasthmatic-like changes in constrictor and relaxant responsiveness exhibited by LPS-stimulated rabbit ASM tissues. As depicted by the relaxation response curves to isoproterenol in Fig. 9B, relative to control control segments, the mean ± SE Rmax value was significantly reduced in LPS-exposed tissues at 27.5 ± 4.5% compared with the value of 50.5 ± 6.2% obtained in the control tissues (P < 0.01). This impaired relaxation responsiveness to isoproterenol was largely pre-

Fig. 8. EMSA analysis of nuclear protein extracts showing NF-κB binding activity in human ASM cells under control conditions (lane 1) and at 20 min after exposure to LPS (0.1 μg/ml) in the absence and presence of pretreatment of the cells for 30 min either with U0126 or with the p38 MAPK activator anisomycin, the latter given alone or in combination with the JNK inhibitor SP-600125. LPS-induced NF-κB binding activity (lane 2) is inhibited in cells pretreated with U0126 (5 μM, lane 3) and in LPS-exposed cells pretreated with anisomycin (50 ng/ml) alone (lane 5) or in combination with SP-600125 (1 μM, lane 6). The EMSA shown is representative of 3 separate experiments. 

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or presence of pretreatment of the tissues for 30 min with anisomycin (50 ng/ml). Incubations were terminated at the times indicated, and cell lysates were prepared using anti-phospho-ERK1/2 and anti-ERK2 antibodies. Data represent means ± SE values from 4 separate experiments.

Discussion

Apart from their well-documented effects in cells comprising the innate immune system, TLRs were found to stimulate the JNK inhibitor SP-600125 (1 μM; data not shown).

Apart from their well-documented effects in cells comprising the innate immune system, TLRs were found to stimulate the JNK inhibitor SP-600125 (1 μM; data not shown).

Our present observations generally concur with those in recent reports demonstrating that LPS elicits activation of the MAPK signaling cascade in various cell types, including ASM cells (4, 28, 30). In this regard, LPS-induced activation of ERK1/2 was previously found to mediate enhanced bradykinin-stimulated accumulation of inositol phosphates and Ca^{2+} mobilization in cultured canine tracheal smooth muscle cells (30). Contrasting with this observation, LPS-induced enhancement of bradykinin-stimulated contractions of murine tracheal smooth muscle segments was found to be largely regulated by activation of the JNK signaling pathway, whereas ERK1/2 activation was not found to play a significant role (4). Our data herein clearly implicate ERK1/2 activation in regulating both the LPS-induced production of IL-6 in human ASM cells and the induction of proasthmatic-like perturbations in agonist-mediated constrictor and relaxant responsiveness in rabbit ASM tissues. These findings are in accordance with earlier reports demonstrating a crucial role for activation of the ERK1/2 signaling pathway in regulating cytokine synthesis by stimulated ASM cells (2, 15, 22, 25). Moreover, our observations fundamentally agree with those in recent studies that demonstrated an important role for ERK1/2 activation in mediating the in vivo airway hyperresponsiveness seen in ovalbumin-sensitized mice (9) as well as the changes in constrictor and relaxant responsiveness observed in rabbit ASM tissues directly stimulated by the dust mite allergen Der p1 (17).

In addition to ERK1/2 signaling, the present observations demonstrated a critical role for NF-κB activation in mediating both IL-6 production and the induced changes in constrictor and relaxation responsiveness in LPS-stimulated ASM. A number of recent studies using different experimental cell models have reported a causal relationship between ERK1/2 signaling and NF-κB activation in LPS-stimulated cells, resulting in the evoked transcription of various proinflammatory genes (40). In accordance with these studies, the present results demonstrated that LPS-induced activation of NF-κB/DNA binding activity was markedly impaired in the presence of ERK1/2 inhibition (Fig. 5A). Moreover, in concert with this observation, we found that ASM cells treated with LPS exhibited an induced association of phosphorylated ERK1/2 with the p65 subunit of NF-κB (Fig. 5B). Accordingly, the present observations clearly implicate a regulatory role for ERK1/2 in a variety of non-bone marrow-derived resident cell types including endothelial cells (3, 41), cardiac myocytes (13, 14), and pulmonary epithelial cells (18, 34). Moreover, it has recently been demonstrated that ASM cells from different species, including humans, also express various TLRs (4, 35). The present results agree with these earlier observations in demonstrating that human ASM cells constitutively express TL4 and TLR9 mRNA transcripts. In contrast to TLR9, activation of TL4 by LPS was found to elicit release of the pleiotropic proinflammatory cytokine IL-6 and to evoke proasthmatic-like changes in ASM constrictor and relaxant responsiveness. Furthermore, these effects were found to be regulated by TL4-induced activation of the ERK1/2 and p38 MAPK signaling pathways, whereby the proasthmatic responses to LPS were attributed to ERK1/2-dependent activation of NF-κB, whereas coactivation of p38 MAPK exerted a homeostatic counterregulatory action by limiting ERK1/2 signal and, hence, the magnitude of NF-κB activation and its mediated changes in ASM responsiveness.
mediating LPS-induced activation of NF-κB in ASM and suggest that phosphorylated ERK1/2 exerts its regulatory action, at least in part, via a direct molecular interaction with the p65 component of NF-κB. Further studies are needed to systematically examine the nature of this interaction and to identify other potential sites of interaction between the ERK1/2 and NF-κB signaling pathways in ASM.

Our data further demonstrated that, relative to ERK1/2 activation, LPS induced a lesser degree of p38 MAPK activation in the human ASM cells and, based on the results provided in Fig. 2A, the calculated ratio of the mean levels of phosphorylated ERK1/2/p38 MAPK amounts to 3.3 in the LPS-exposed cells compared with a ratio of 1.3 pertaining to the ISS-ODN-treated cells. In accordance with this enhanced activation of ERK1/2 relative to p38 MAPK, LPS also induced relatively enhanced IL-6 release compared with that elicited by ISS-ODN (Fig. 1B). Moreover, the magnitude of LPS-induced release of IL-6 was found to be essentially unaltered in the presence of p38 MAPK inhibition (Fig. 2B). Contrasting with this finding in the cultured human ASM cells, LPS-induced changes in constrictor and relaxant responsiveness in rabbit ASM tissues were found to be relatively more pronounced in the presence of p38 MAPK inhibition (Figs. 6 and 7), implicating a regulatory role for p38 MAPK activation in the LPS-stimulated ASM tissues. At first glance, this disparity may be explained by species-dependent differences in either the degree or responsivity to p38 MAPK activation in LPS-stimulated ASM. In this context, given our extended observations demonstrating a pronounced inhibitory effect of anisomycin on LPS-induced NF-κB binding activity in the human ASM cells (Fig. 8), it is reasonable to speculate that the observed species-dependent differences in the effects of p38 MAPK inhibition most likely reflected differences in the degree of p38 MAPK activation between the LPS-stimulated human ASM cells vs. rabbit ASM tissues. Notwithstanding this issue, the observed changes in ASM responsiveness accompanying p38 MAPK inhibition (Figs. 6 and 7) and those obtained in the presence of p38 MAPK activation with anisomycin (Fig. 9B) provide complementary evidence that p38 MAPK activation attenuates the ERK1/2-dependent/NF-κB-mediated proasthmatic-like action of LPS on ASM responsiveness. This notion is further supported by the results demonstrating that the magnitude of LPS-stimulated p38 MAPK activity was increased in ASM cells pretreated with a p38 MAPK inhibitor (Fig. 5) and that LPS-induced NF-κB binding activity was inhibited in ASM cells pretreated with anisomycin (Fig. 8). This observed action of p38 MAPK is similar to that recently reported in studies conducted on T lymphocytes (29) and melanoma cells (26) subjected to ultraviolet irradiation where p38 activation was found to inhibit phosphorylation of ERK and JκB proteins and, hence, NF-κB-dependent gene transcription.

In further considering the present findings, our observations are consistent with those in a recent study that demonstrated qualitatively similar effects of p38 MAPK activation in preventing the induced changes in ASM responsiveness exhibited by rabbit ASM tissues exposed to the dust mite allergen Der p1 (17). In the latter study, we identified that p38 MAPK exerts its modulatory effects on ASM responsiveness by inhibiting ERK1/2 signaling and thereby attenuates the proinflammatory effects of ERK1/2 activation on ASM responsiveness (17). Although the mechanism(s) underlying this inhibitory action of p38 MAPK remains to be identified, it is relevant to note that a one-way cross talk between p38 MAPK and ERK1/2 was recently demonstrated where phosphorylated p38α was found to couple with ERK1/2 and thereby sterically block ERK1/2 phosphorylation by MEK1/2 (45) and possibly also act via a protein kinase that lies upstream of MEK1/2 (39). Our results provide evidence that confirms this cross talk mechanism, given the observations that activation of p38 MAPK with anisomycin largely prevented both the LPS-induced enhanced phosphorylation of ERK1/2 (Fig. 9A) and changes in ASM responsiveness (Fig. 9B), whereas anisomycin had no effect in control ASM.

In conclusion, the present study investigated the role and mechanisms of action of TLR activation in regulating ASM function. The results provide new evidence demonstrating that 1) human ASM cells constitutively express TLR4 and TLR9 mRNAs; 2) whereas TLR9 stimulation has little effect, stimulation of TLR4 with LPS elicits a significant increase in IL-6 release that is attributed to activation of the ERK1/2 signaling pathway which, in turn, is coupled to activation of NF-κB; 3) LPS also elicits proinflammatory changes in agonist-mediated constrictor and relaxant responsiveness in rabbit ASM tissues; and 4) the latter effects are also attributed to ERK1/2 and NF-κB activation, whereas coactivation of p38 MAPK serves to limit homeostatically the magnitude of LPS-induced ERK1/2 signaling and changes in ASM responsiveness. Collectively, these observations provide new information supporting the concept that, apart from the role played by immune surveillance cells in the lung, activation of TLR4 in ASM represents an important mechanism by which pulmonary exposure to LPS evokes expression of the airway asthmatic phenotype.

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