Inhibitory Influence of Protease-Activated Receptor 2 and E-Prostanoid Receptor Stimulants in Lipopolysaccharide Models of Acute Airway Inflammation

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ABSTRACT
Protease-activated receptors (PARs) are widely expressed throughout the respiratory tract, and PAR2 has been investigated as a potential drug target for inflammatory airway diseases. The primary focus of this study was to determine the extent to which PAR2-activating peptides modulate lipopolysaccharide (LPS)-induced airway neutrophilia in mice and establish the underlying mechanisms. Intranasal administration of LPS induced dose- and time-dependent increases in the number of neutrophils recovered from bronchoalveolar lavage (BAL) fluid of mice. Coadministration of the PAR2-activating peptide f-LIGRL inhibited LPS-induced neutrophilia at 3 and 6 h after inoculation. PAR2-mediated inhibition of LPS-induced neutrophilia was mimicked by prostaglandin E2 (PGE2) and butaprost (selective E-prostanoid (EP2) receptor agonist), and blocked by parecoxib (cyclooxygenase 2 inhibitor) and 6-isopropoxy-9-oxoxanthene-2-carboxylic acid (AH6809) (EP1/EP2 receptor antagonist). PAR2-activating peptides also blunted early increases in the levels of the key neutrophil chemoattractants keratinocyte-derived chemokine and macrophage inflammatory protein 2 (MIP-2) in the BAL of LPS-exposed mice. However, neither PAR2-activating peptides nor PGE2 inhibited LPS-induced generation of MIP-2 in cultures of primary murine alveolar macrophages. In summary, PAR2-activating peptides and PGE2 suppressed LPS-induced neutrophilia in murine airways, independently of an inhibitory action on MIP-2 generation by alveolar macrophages.

Introduction
Protease-activated receptors (PAR1, PAR2, PAR3, and PAR4) are a unique family of G protein-coupled receptors (Steinhoff et al., 2005). PARs are targets of specific proteases, which cleave the extracellular N terminus of the PAR, exposing a newly formed tethered ligand that activates the receptor (Schmidlin and Bunnett, 2001). PAR1, PAR2, and PAR4 are also targeted and activated by small peptide sequences that mimic the first five to eight amino acids of the tethered ligand (Devlin et al., 2007). For example, peptide sequences such as SLIGRL and f-LIGRL activate murine PAR2 (Kawabata et al., 2004). f-LIGRL seems more suited to in vivo investigations because it is a more potent activator of PAR2 than SLIGRL and is resistant to degradation by aminopeptidases (Kawabata et al., 2004). Partially scrambled analogs of SLIGRL and f-LIGRL, such as LSIGRL and f-LRGIL, do not activate PAR2 receptors and are regularly used as control peptides in experiments involving PAR2-activating peptides (PAR2-APs) (Abey et al., 2006).

PARs are widely expressed, with high levels of expression reported in the cardiovascular, gastrointestinal, and respiratory systems. Of specific relevance to this study, PAR2 is abundantly expressed by many resident structures within the airways, including airway epithelial cells, fibroblasts, myocytes, sensory neurons, endothelial cells, and vascular smooth muscle (Cocks et al., 1999; Peters and Henry, 2009). PAR2 is also expressed on a variety of immune cells including macrophages, lymphocytes, neutrophils, eosinophils, and mast cells, suggesting a role for PAR2 in inflammatory processes (Steinhoff et al., 2005). PAR2 expression is increased in asthmatic bronchi and airways exposed to respiratory tract viruses, allergens, inflammatory cytokines, and smoke (Knight et al., 2001; Lan et al., 2004; Freund-Michel and Frossard, 2006). However, the functional significance of increased PAR2 expression in inflammatory lung disorders is controversial because there is evidence of PAR2 mediating...
both proinflammatory and anti-inflammatory activities (Peters and Henry, 2009).

Activators of PAR2 have been shown to variably influence the level of bronchomotor tone, the development of airways' hyperresponsiveness, and the extent of inflammatory cell influx in animal models of respiratory tract disease induced by allergens, viruses, and bacterial products (Khoucha et al., 2009; Peters and Henry, 2009). For example, PAR2-APs reduced the airway inflammatory responses induced by the bacterially derived product lipopolysaccharide (LPS) (Moffatt et al., 2002). However, the underlying mechanism for SLIGRL-induced inhibition of LPS-induced airway neutrophilia has not been determined, so elucidating this mechanism is a primary focus of the current study. One possibility to be explored is that PAR2-APs generate prostanoids, such as prostaglandin E2 (PGE2), which exert downstream anti-inflammatory effects within the lungs. Evidence supporting this mechanism includes the finding that PAR2-APs elevate PGE2 levels in respiratory epithelial cells, intact airway segments, and in vivo (Lan et al., 2001; De Campo and Henry, 2005; Kawao et al., 2005). Furthermore, PGE2 has been reported to produce a wide range of anti-inflammatory effects within the airways (Pavord and Tattersfield, 1995; Vancheri et al., 2004), including inhibition of LPS-induced airway neutrophilia (Goncalves de Moraes et al., 1996). This may be a novel therapeutic approach in chronic obstructive pulmonary disease and severe asthma, where neutrophils are thought to play an important role in exacerbations of disease (Beek and Beier, 2006).

An initial aim of this study was to examine the inhibitory influence of a range of doses of the PAR2-APs SLIGRL and f-LIGRL (and the control peptide LSIGRL) on the time course of LPS-induced neutrophilia in mice. The putative mediator role of endogenous PGE2 in these inhibitory processes was subsequently investigated pharmacologically by pretreatment of mice with cyclooxygenase (COX) inhibitors and prostanoid receptor antagonists. These were complemented by experiments that determined the influence of exogenous PGE2 and an EP2 receptor-selective agonist butaprost on LPS-induced neutrophilia. Additional mechanistic studies were conducted by using isolated alveolar macrophages to determine the influence of PAR2-APs and PGE2 on the levels of cytokines and chemokines thought to play an important role in the recruitment of neutrophils to LPS-exposed mouse lungs, including tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-2 (MIP-2).

**Materials and Methods**

**Materials.** The PAR2-APs SLIGRL and f-LIGRL and the control peptides LSIGRL and f-LIRGL were synthesized by Proteomics International (Perth, Australia). LPS (Escherichia coli strain 0127:B8), butaprost, indomethacin, and cis-2-(diphenylmethyl)-N-[(2-isodophenyl) methyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate salt (L-703606) were purchased from Sigma-Aldrich (St. Louis, MO), and parecoxib was from Pfizer (Paris, France). PGE2 was purchased from Cayman Chemical (Ann Arbor, MI). Anti-mouse TNF-α antibody, goat IgG isotype control, and enzyme-linked immunosorbent assay kits to measure levels of keratinocyte-derived chemokine (KC) and MIP-2 were purchased from R&D Systems (Minneapolis, MN).

**Animals.** Male BALB/c mice (specific pathogen free) at 8 to 10 weeks old, weighing approximately 20 g, were purchased from the Animal Resource Centre (Murdoch, Australia). Animals were housed on a 12-h light/12-h dark cycle and given sterile food and water ad libitum. Experiments were performed in accordance with the National Health and Medical Research Council guidelines as outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004).

**Intranasal Inoculation.** Mice were lightly anesthetized with methoxyflurane vapor and held upright, and a 20-μl aliquot of inoculum was applied to each of the external nares. As oblige nose breathers, mice inhaled the inoculum into their airways. The inoculum contained either LPS or vehicle (sterile saline). LPS derived from the cell wall of E. coli strain 0127:B8 was selected for use because this strain previously had been demonstrated to induce airway inflammation in BALB/c mice (Moffatt et al., 2002). Appropriate LPS doses (0.3–30 μg/mouse) and periods of exposure (0–24 h) were determined from dose–response and time course studies. In selected experiments, the intranasal inoculum also contained a PAR2-AP (SLIGRL, 25–400 μg/mouse; f-LIGRL, 3–30 μg/mouse) or an EP receptor agonist (PGE2, 10 μg/mouse; butaprost, 10 or 50 μg/mouse). The partially scrambled peptide LSIGRL (400 μg/mouse), which does not activate PAR2, was administered in control experiments.

In mechanistic studies, mice were administered a cyclooxygenase inhibitor (indomethacin, 1 mg/kg i.p. or parecoxib, 1 mg/kg i.p.), an EP receptor antagonist [6-isopropoxy-9-oxoanthene-2-carboxylic acid (AH6809); 5 mg/kg i.p.] or a NK1 receptor antagonist (L-703606; 3 mg/kg i.p.) 1 h before intranasal peptide or LPS inoculation.

**Bronchoalveolar Lavage.** Mice were euthanized with pentobarbitone sodium (160 mg/kg i.p.), and the trachea was intubated with a Teflon-coated cannula. Lungs were lavaged with 6 × 0.5-ml aliquots of ice-cold, pyrogen-free phosphate-buffered saline, pH 7.4, and the resultant bronchoalveolar lavage fluid (BALF) was centrifuged (1500 rpm, 7 min, 4°C). The cell pellet was resuspended in sterile red blood cell lysis buffer (Tris ammonium chloride buffer; 0.83% NH4Cl in Tris, pH 7.65) for 8 min at room temperature. After washing, BAL cells were resuspended in phosphate-buffered saline, pH 7.4 with 1% bovine serum albumin or complete RPMI medium 1640 supplemented with 10% fetal calf serum, 100 μg/ml gentamycin, and 2 mM Glutamax (Invitrogen, Carlsbad, CA). Total leukocyte number was determined by using a hemocytometer, and cell viability (always >95%) was established by trypan blue (0.4%) exclusion. Cytospin preparations of BAL cells were stained with Rapid I, II (Amber Scientific, Perth, Australia) and differential cell counts of 400 cells were performed at ×40 magnification (Olympus BX-50 light microscope; Olympus, Tokyo, Japan). In some instances, cell numbers in PAR2-AP-treated mice are expressed as a percentage reduction of the mean recruitment of cells in the absence of drug.

**Alveolar Macrophage Culture.** Primary alveolar macrophages were isolated from BALF by a simple adhesion method. BALF from 20 mice was pooled and centrifuged, and the cell pellet was purged of red blood cells by Tris ammonium chloride treatment as described above. The washed cell pellet was resuspended in 1.0 ml of complete RPMI medium, a cell count was performed, and the cells were diluted such that 4 × 104 viable cells were plated per well in 100 μl of medium in a 96-well plate (NUNC A/S, Roskilde, Denmark). Macrophages were allowed to adhere for 1 h at 37°C in a 5% CO2 humidified incubator. Nonadherent cells and debris were removed by washing. The adherent macrophages were cultured in 100 μl of fresh complete medium and treated with either the PAR2-APs SLIGRL (1, 10, or 100 μM/well), f-LIGRL (20 μM), their scrambled control counterparts LSIGRL (100 μM) and f-LIRGL (20 μM), or PGE2 (10 μM) 10 min before stimulation with LPS (10 ng/ml). Combinations were tested at least in triplicate per plate, and each individual experiment was repeated at least four separate occasions. To reduce the metabolic rate of the macrophages and hence slow down the very rapid synthesis of cytokines, at various time points (separate experiments) the culture plates were put on ice and 100 μl of ice-cold medium was added per well. Culture supernatants were quickly harvested and stored at –80°C until used for MIP-2, KC, TNF-α, and PGE2. In some experiments, cultures were treated with an anti-TNF-α antibody 10 min before exposure to LPS.
Chemokine and Cytokine Enzyme-Linked Immunosorbent Assays. Levels of the chemokines MIP-2 and KC, the cytokine TNF-α, and the prostaglandin PGE₂ were determined in the culture supernatants of peptide- or PGE₂-treated, LPS-stimulated primary alveolar macrophages. MIP-2, KC, and TNF-α enzyme immunoassay kits were purchased from R&D Systems, and the PGE₂ kit was from Cayman Chemical. All supernatants were assayed according to individual kit instructions.

Statistical Analyses. Data are presented as the mean ± S.E.M. of the group. All data were checked for normality and equal variance before statistical analysis. Statistical analysis was performed by using a commercial software package (SigmaStat version 3.5; Systat Software, Inc., San Jose, CA). Statistical analyses for multiple comparisons were completed by using analysis of variance with an appropriate post hoc test. All pairwise comparisons were completed by using the Holm-Sidak method. A $p$ value < 0.05 was considered statistically significant.

Results

LPS Model of Acute Airway Inflammation. As expected, intranasal inoculation of mice with LPS caused significant dose- and time-dependent increases in total BAL cell number (Fig. 1, A and D). The influx of cells into the airways occurred rapidly, with the total BAL cell number recovered at 3 h after inoculation being 6.6-± 0.8-fold higher in LPS-treated mice than in control mice ($p < 0.001$; Fig. 1A), with the peak increase occurring between 3 and 24 h after inoculation. Up until at least 48 h after inoculation, the increase in total BAL cell number in LPS-treated mice was almost entirely caused by an increase in the number of neutrophils (Fig. 1B). Thereafter, the number of neutrophils steadily declined to levels that were only marginally elevated at 72 h (Fig. 1B). The number of BAL macrophages remained relatively constant during the first 48 h after administration of LPS, but were significantly elevated at 72 h (Fig. 1C).

Over the dose range of 0.3 to 30 μg of LPS/mouse, a strong positive relationship was observed between dose and total BAL cell number (Fig. 1D) and dose and BAL neutrophil number (Fig. 1E) at 6 h after inoculation. In contrast, the number of macrophages recovered from BAL fluid (at 6 h after inoculation) was independent of the dose of LPS administered (Fig. 1F).
summary, intranasal administration of LPS to mice caused a profound inflammatory response in the airways, dominated by a transient and reversible neutrophilia.

PAR2-APs Suppressed LPS-Induced Increases in BAL Neutrophil Numbers. To determine the influence of PAR2-APs on the time course of LPS-induced neutrophilia, groups of mice were intranasally inoculated with a sterile solution containing 10 μg of LPS, with or without SLIGRL. In these experiments, a dose of 400 μg of SLIGRL/mouse was used because it had been reported previously to be effective in a murine model of airway inflammation (Moffatt et al., 2002). At 3 h after inoculation, BAL fluid recovered from SLIGRL-treated LPS mice had 78 ± 4% fewer neutrophils than LPS-only mice (Fig. 2A; p < 0.01). However, SLIGRL did not significantly reduce the number of neutrophils in BAL fluid recovered from LPS mice at either 6 or 24 h after inoculation (Fig. 2A).

Additional studies were conducted with f-LIGRL, an aminopeptidase-resistant, longer-acting PAR2-AP (Kawabata et al., 2004). A dose of 30 μg of f-LIGRL reduced LPS-induced BAL neutrophil numbers by 77 ± 7% at 3 h after inoculation and 35 ± 2% at 6 h after inoculation (Fig. 2B; p < 0.001). However, f-LIGRL did not significantly inhibit the number of BAL neutrophils recovered from LPS-exposed mice at 24 h after inoculation (Fig. 2B). In summary, f-LIGRL was able to reduce LPS-induced BAL neutrophil numbers for a longer period than SLIGRL, although not over the entire time course of LPS-induced inflammation.

In subsequent studies, the dose-response relationship for SLIGRL- and f-LIGRL-induced inhibition of BAL neutrophil numbers was determined at 3 h after inoculation (Fig. 3). Administration of 25, 100, and 400 μg of SLIGRL/mouse...
reduced the numbers of BAL neutrophils recovered from LPS-exposed mice by 36 ± 3, 47 ± 2, and 74 ± 2%, respectively (Fig. 3A; p < 0.001). f-LIGRL also produced a dose-dependent inhibition of LPS-induced BAL neutrophil numbers, with 10 and 30 μg of f-LIGRL/mouse reducing numbers of BAL neutrophils by 37 ± 6 and 65 ± 4%, respectively (Fig. 3B; p < 0.05). SLIGRL or f-LIGRL did not produce any alteration in the BAL cell profile of control mice (Fig. 3, A and B). The partially scrambled peptide sequence LSIGRL (400 μg/mouse) did not significantly alter BAL neutrophil numbers in either LPS-treated or control mice (Fig. 3C).

**Cyclooxygenase Inhibitors Blocked Responses to PAR2-APs.** To determine whether cyclooxygenase products mediated the inhibitory effects of PAR2-APs on BAL neutrophilia, a nonselective COX inhibitor, indomethacin, was administered intraperitoneally to mice 1 h before inoculation with LPS and/or PAR2-APs. As expected, 30 μg/mouse of f-LIGRL reduced LPS-induced BAL neutrophil numbers (by 77 ± 7%; Fig. 5A). However, f-LIGRL did not significantly reduce BAL neutrophil numbers in indomethacin-treated, LPS-exposed mice (Fig. 4A).

Parecoxib, a selective COX-2 inhibitor, was used to determine whether the COX-2 isozyme played a primary role in f-LIGRL-induced inhibition of LPS-induced neutrophilia. In these studies, 30 μg of f-LIGRL/mouse reduced the number of BAL neutrophils recovered from LPS-exposed mice by 68 ± 9% (p < 0.001; Fig. 4B). However, in LPS-exposed mice treated with parecoxib f-LIGRL failed to significantly reduce BAL neutrophil numbers (Fig. 4B). Parecoxib did not significantly alter the numbers of BAL neutrophils in saline- or LPS-treated mice in the absence of f-LIGRL. Thus, inhibition of COX-2 attenuated the suppressive effect of PAR2-APs on LPS-induced neutrophilia.

**EP Receptor Agonists Mimicked, and EP Receptor Antagonists Inhibited, Responses to PAR2-APs.** PGE2 is a product of COX-2 that mediates a range of effects induced by PAR2-APs. Consistent with this, PGE2 reduced the number of LPS-induced BAL neutrophils recovered at 3 h after inoculation by 54 ± 5% (Fig. 5A; p < 0.001). Likewise, a selective EP2 receptor agonist, butaprost, produced an 83 ± 5% reduction in neutrophils recovered from LPS-treated mice (Fig. 5B; p < 0.001). In additional studies, a mixed EP1/EP2/DP receptor antagonist (AH6809) significantly inhibited the effects of f-LIGRL on LPS-induced airway neutrophilia.
In summary, these findings are consistent with the postulate that f-LIGRL-induced suppression of LPS-induced airway neutrophilia was mediated via PGE2 and its activation of EP2 receptors.

NK1 Receptor Antagonists Did Not Inhibit Responses to PAR2-APs. As PAR2-APs have been reported to activate NK1 receptors (Abey et al., 2006), experiments were performed to determine whether a NK1 receptor antagonist, L-703606, altered f-LIGRL-induced decreases in LPS-induced BAL neutrophil numbers. Similar to previous observations, LPS caused a marked increase in the number of BAL neutrophils, and f-LIGRL reduced this effect by 65 ± 3% (Fig. 6B). The NK1 receptor antagonist L-703606 (3 mg/kg i.p. 1 h before LPS and/or peptides) did not significantly alter the number of BAL neutrophils recovered in response to LPS in the presence or absence of f-LIGRL (Fig. 6B).

f-LIGRL Suppressed the Early LPS-Induced Increases in BAL Fluid Levels of KC and MIP-2. Neutrophil recruitment into the LPS-exposed airways is preceded and promoted by increases in the levels of chemokines such as KC and MIP-2. Consistent with this, the levels of KC and MIP-2 in BAL fluid recovered 1 h after exposure to LPS were 26- and 4.2-fold higher, respectively, than in saline-exposed mice (Fig. 7A). Of particular interest, BAL fluid recovered from LPS-exposed mice that received f-LIGRL (30 μg/mouse) had levels of KC and MIP-2 40 to 45% lower than that recovered from mice receiving LPS alone (P < 0.01; Fig. 7A). However, f-LIGRL did not suppress the elevated levels of KC and MIP-2 detected in BAL fluid recovered 3 h after LPS (Fig. 7B).

PAR2-APs and PGE2 Did Not Inhibit LPS-Induced MIP-2 Production in Murine Alveolar Macrophages. To further investigate the underlying mechanisms for PAR2- and PGE2-mediated suppression of chemokine levels in vivo, additional studies were conducted with primary cultures of murine alveolar macrophages. LPS induced profound time- and concentration-dependent increases in MIP-2 levels in isolated alveolar macrophages (Fig. 8, A and B). However, LPS-induced increases in MIP-2 levels were not significantly inhibited by f-LIGRL (or control peptide f-LRGIL; Fig. 8C), SLIGRL (or control peptide LSIGRL; Fig. 8D), or PGE2 (Fig. 8E).

TNF-α Played a Minor Role in LPS-Induced Increases in MIP-2 Production in Murine Alveolar Macrophages. LPS-exposed alveolar macrophages generated high levels of TNF-α (Fig. 9A). LPS-induced increases in TNF-α were unaffected by f-LIGRL (Fig. 9A), but were significantly inhibited by either 1 or 10 μM PGE2 (Fig. 9B). Despite the high levels of TNF-α produced in response to LPS, murine TNF-α was a very weak activator of MIP-2 generation in murine alveolar macrophages (Fig. 9C).
sistent with this, a TNF-\(\alpha\)-absorbing monoclonal antibody did not significantly reduce LPS-induced MIP-2 generation (Fig. 9C).

**Effect of SLIGRL on PGE\(_2\) Levels in Cultures of Alveolar Macrophages.** Exposure of alveolar macrophage cultures to SLIGRL or control peptide (LSIGRL) for up to 2 h did not induce any measurable increase in PGE\(_2\) levels (Fig. 10). The elevated levels of PGE\(_2\) observed in SLIGRL-treated alveolar macrophage cultures at 24 h (Fig. 10) were not significantly different from levels observed in response to either LSIGRL (Fig. 10) or vehicle (data not shown).

**Discussion**

In the current study, intranasal administration of PAR\(_2\)-APs produced dose- and time-dependent inhibition of LPS-induced pulmonary neutrophilia in mice. Additional in vivo and in vitro mechanistic studies indicate that this process involves PAR\(_2\)-mediated, COX-2-dependent production of PGE\(_2\), which suppresses airway neutrophilia independently of a direct inhibitory action on MIP-2 generation by alveolar macrophages.

Inhalation of bacterial-derived LPS produces a profound immune response characterized by a rapid recruitment of neutrophils into the lung. In the current study, LPS-induced increases in BAL neutrophils were evident within 3 h of intranasal inoculation, peaked between 6 and 24 h after inoculation, and had resolved by 72 h after inoculation. Furthermore, the number of neutrophils recovered from the BAL fluid of LPS-exposed mice was directly related to the dose of LPS administered. Dose- and time-dependent increases in LPS-induced neutrophilia have been reported previously (Szarka et al., 1997; Bozinovski et al., 2004), as has the rebound increase in macrophages observed at 72 h after inoculation (Moffatt et al., 2002; Bozinovski et al., 2004), which is required to phagocytose the large numbers of neutrophils undergoing apoptosis (Cox et al., 1995).

In mice, the magnitude of LPS-induced neutrophilia was variably inhibited by the intranasal administration of PAR\(_2\)-APs. At 3 h after inoculation, both SLIGRL and f-LIGRL inhibited BAL neutrophilia in a dose-dependent manner, and as expected for a PAR\(_2\)-mediated response, f-LIGRL was more potent than SLIGRL (Kawabata et al., 2004). The du-
ration of PAR2-mediated inhibition of LPS-induced neutrophilia was also peptide-dependent. Whereas both SLIGRL and f-LIGRL inhibited LPS-induced airway neutrophilia at 3 h after inoculation, only f-LIGRL was effective 6 h after inoculation. Neither PAR2-AP inhibited LPS-induced neutrophilia at 24 h after inoculation. A likely explanation for these differences in duration of effect is the greater resistance of f-LIGRL to peptide degrading aminopeptidases (Kawabata et al., 2004). The finding that SLIGRL inhibited LPS-induced neutrophilia is consistent with a previous report (Moffatt et al., 2002), although differences in the onset and duration of action were evident. In contrast to the current study, Moffatt and coworkers found that SLIGRL-induced inhibition of LPS-induced pulmonary neutrophilia was considerably delayed, with no significant reduction in neutrophils observed at either 3 or 6 h after inoculation. The reasons for these differences are unclear, but cannot be explained on the basis of the strain of mouse used, the method used for peptide and LPS administration, or the dose of SLIGRL used, because these were similar in both studies.

In addition to PAR2s, PAR2-APs are known to activate NK1 receptors (Abey et al., 2006). However, f-LIGRL-induced reductions in LPS-induced neutrophilia were unaffected by administration of a NK1 receptor antagonist, indicating that these receptors were not involved in PAR2-AP-induced reductions in LPS-induced neutrophilia. In summary, data obtained from studies using control peptides (LSIGRL, f-LRGIL) and NK1 receptor antagonists indicate that f-LIGRL- and SLIGRL-induced reductions in LPS-induced neutrophilia are mediated via activation of PAR2.

Several PAR2-mediated effects within the airways have been attributed to the generation of prostanoids such as PGE2. For example, PAR2-mediated relaxation of airway smooth muscle and inhibition of allergic inflammation both were blocked by inhibitors of cyclooxygenase such as indomethacin (Cocks et al., 1999; Lan et al., 2001; De Campo and Henry, 2005). Likewise, in the current study, indomethacin blocked f-LIGRL-induced inhibition of pulmonary neutrophilia in LPS-exposed mice. A similar effect was produced by parecoxib, indicating the particular involvement of COX-2 in this process. Consistent with the likely mediator role played by endogenous PGE2 in f-LIGRL-induced effects, we observed that the direct intranasal application of exogenous PGE2 also inhibited neutrophilia in LPS-exposed mice. These data concur with earlier findings that intranasal application of PGE2 inhibited neutrophil recruitment in response to LPS (Goncalves de Moraes et al., 1996). Furthermore, intranasal administration of the selective EP2 receptor agonist butaprost inhibited the increase in BAL neutrophils observed in LPS-exposed mice. A role for the EP2 receptor in PAR2-mediated inhibition of LPS-induced neutrophilia is also supported by the findings that an EP2 receptor antagonist AH6809 inhibited f-LIGRL-induced effects in the LPS model. Together, these data indicate a role for EP2 receptors in PAR2-mediated reductions in LPS-induced neutrophilia, although it is possible that other
EP receptors and COX products are involved in PAR2-mediated anti-inflammatory effects. The chemokines MIP-2 and KC are functionally related to human IL-8 and play a key role in the recruitment of neutrophils in LPS-exposed rodents (Lee et al., 1995; Schmal et al., 1996; De Filippo et al., 2008). In line with this chemottractant role, markedly elevated levels of MIP-2 and KC were detected in the BAL fluid of LPS-exposed mice before significant increases in the number of neutrophils. Moreover, the levels of both chemokines were significantly lower in LPS-exposed mice treated with f-LIGRL. To investigate whether direct inhibition of MIP-2 release by alveolar macrophages underpinned the inhibitory effect of PAR2-APs on LPS-induced neutrophilia, additional in vitro studies were performed with primary cultures of alveolar macrophages, a significant cellular source of neutrophilic chemokines (Merrill et al., 1980; Goodman et al., 1998; Amano et al., 2000; Thorley et al., 2007). In these studies, LPS produced profound concentration- and time-dependent increases in MIP-2 levels in murine alveolar macrophage cultures. Of particular relevance, PGE2 or PAR2-APs (SLIGRL, f-LIGRL) did not significantly affect LPS-induced increases in MIP-2 levels in murine alveolar macrophage cultures. Although the effect of PAR2-APs on MIP-2 release from alveolar macrophages has not been reported previously, the findings with PGE2 are consistent with several previous reports that this prostaglandin failed to inhibit IL-8 release from human alveolar macrophages (Strieter et al., 1989; Standiford et al., 1992), despite an inhibitory action on human blood monocytes (Standiford et al., 1992). Thus, the inhibitory effect of PAR2-APs and PGE2 on LPS-induced increases in BAL fluid MIP-2 levels and neutrophil numbers observed in vivo cannot be readily explained by direct inhibition of MIP-2 generation by LPS-exposed alveolar macrophages.

The cytokine TNF-α has also been implicated in LPS-induced pulmonary neutrophilia. For example, the lung neutrophil influx observed after LPS exposure in mice could be suppressed by neutralizing anti-TNF-α antibodies (Goncalves de Moraes et al., 1996) or by using mice that were deficient in receptors for TNF-α (and IL-1β) (Mizgerd et al., 2001; Jones et al., 2005). Thus, processes that inhibit LPS-induced TNF-α generation may reduce neutrophil influx. In this context, we observed that LPS-induced generation of TNF-α by murine alveolar macrophages was suppressed by PGE2 but not by f-LIGRL. This raises the possibility that PAR2-APs promote the synthesis and release of PGE2, which then inhibits LPS-induced generation of TNF-α by alveolar macrophages (Smith et al., 2004; Ratcliffe et al., 2007). The cellular source of PGE2 is likely to be the alveolar macrophage because these cells did not produce PGE2 in response to PAR2-APs, but may be airway epithelial cells that are known to generate PGE2 rapidly in response to PAR2-APs (Lan et al., 2001; Kawai et al., 2005; Saleh et al., 2008). Although it is currently unclear how TNF-α might mediate LPS-induced neutrophil influx, one possibility is that TNF-α produced by LPS-exposed alveolar macrophages stimulates the generation of neutrophilic chemokines such as MIP-2 and IL-8 (Thorley et al., 2007). However, LPS-induced generation of TNF-α was unlikely to play a major role in MIP-2 production by alveolar macrophages because inhibiting the actions of TNF-α with a blocking monoclonal antibody did not suppress LPS-induced generation of MIP-2, and exogenous muc-
PAR2 Activators Blunt LPS-Induced Neutrophilia in Mouse Lung


T-lymphocytes.

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