Mechanisms of immune activation during infection
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CHAPTER 5

P38 Mitogen Activated Protein Kinase is involved in the downregulation of granulocyte CXC chemokine receptors 1 and 2 during human endotoxemia


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Chapter 5

Abstract

Chemokine receptors CXC receptor (CXCR) 1 and 2, and their ligands interleukin (IL)-8 and growth related oncogene (GRO)-α are principal regulators of neutrophil activation and migration. To investigate the role of p38 mitogen activated protein kinase (MAPK) in the regulation of CXCR expression during an inflammatory response in vivo, 24 healthy volunteers received an intravenous injection with lipopolysaccharide (LPS) preceded (-3 hours) by a specific p38 MAPK inhibitor (BIRB 796 BS) in a high dose (600 mg), a low dose (50 mg), or placebo. The LPS-induced reduction of neutrophil CXCR 1 and 2 expression, as determined by fluorescence-activated cell sorter analysis, was inhibited in volunteers receiving the high dose of the p38 MAPK inhibitor. The kinase inhibitor also dose-dependently diminished the LPS-induced rises in plasma IL-8 and GRO-α levels. These results indicate a principal role for p38 MAPK in regulating factors essential for neutrophil activation and chemotaxis in vivo.
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**Introduction**

Neutrophil migration is a hallmark of many acute and chronic inflammatory diseases. In many types of infection, an influx of neutrophils and the subsequent production of reactive oxygen species and proteolytic enzymes form an important line of defense in the innate immune response to invading microorganisms (1). Granulocyte activation and migration to the site of infection is regulated by CXC chemokines, a family of small proteins with strong chemotactic activity. Members of the CXC chemokine family that stimulate granulocyte function include interleukin (IL)-8, growth-related oncogenes (GRO)-α, GRO-β, and GRO-γ; and epithelial-derived neutrophil attractant (ENA)-78 (1). Granulocytes express 2 types of CXC chemokine receptors (CXCR) that interact with these mediators: CXCR1, which exclusively binds IL-8, and CXCR2, which binds IL-8, GROs and ENA-78.

In general, granulocytes respond to infectious and inflammatory agents with a downregulation of surface CXCR1 and 2 (2). In vivo, CXCR1 and CXCR2 expression on granulocytes is reduced during chronic lower respiratory tract infection, and in pulmonary tuberculosis, human immunodeficiency virus (HIV) infection and experimental human endotoxemia (2-4), whereas during sepsis only CXCR2 expression is decreased (5). However, little is known about the intracellular mechanisms involved in the regulation of CXCR expression.

Recently, we found that a member of the Mitogen Activated Protein Kinase (MAPK) family, p38 MAPK, is involved in regulating CXCR2 surface expression on granulocytes in vitro (2). MAPKs are important intracellular transducers of inflammatory signals. Specifically, p38 MAPK plays a cardinal role in regulating stress-induced events, such as cytokine production during inflammation (6). P38 MAPK is activated upon stimulation of neutrophils with LPS (7). Inhibition of p38 MAPK diminishes IL-8 production by monocytes, granulocytes and endothelial cells (8, 9). Thus, in vitro evidence supports a role for p38 MAPK in regulating CXC receptor and ligand expression.

Little is known about the role of p38 MAPK in regulation of CXCR or ligand expression in vivo. In the present study we extend our previously reported in vitro data (2) and show an
important role for p38 MAPK in regulating the expression of granulocyte CXCR1 and CXCR2 and their ligands GRO-α and IL-8 during systemic inflammation in vivo.

Materials and Methods

LPS administration to humans in vivo
This study was a randomized, double-blind, placebo-controlled experiment performed simultaneously with a study examining the effect of BIRB 796 BS on LPS-induced cytokine release and neutrophil activation (10). Briefly, LPS (from E. coli, lot G; United States Pharmacopeial Convention, Rockville, MD; 4 ng/kg body weight) was administered as a bolus intravenous injection to 24 healthy male volunteers (mean age 22 years, range 19-29). Three hours prior to infusion of LPS, the p38 MAPK inhibitor BIRB 796 BS (600 mg n=8, 50 mg n=8) or placebo (n=8) was administered orally in 15 ml of polyethylene glycol 400. BIRB 796 BS is a highly selective and potent p38 MAPK inhibitor developed by Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT (11). Blood samples were obtained before administration of BIRB 796 BS or placebo (t = -3 h), directly before LPS administration (t = 0 h), then 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 8, 10, and 24 hours after LPS administration. The study was approved by the institutional research and ethics committees and written informed consent was obtained from all subjects prior to enrollment.

Cytokine measurements
IL-8 and GRO-α were determined in EDTA-anticoagulated plasma by specific ELISA’s according to the manufacturers’ instructions. (IL-8: Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, the Netherlands; GRO-α: R&D Systems, Abingdon, UK).

FACS analysis
Leukocyte counts and differentials were determined in EDTA-anticoagulated blood using a Stekker analyzer (counter STKS. Coulter counter, Bedfordshire, United Kingdom). Expression of CXCR1 and CXCR2 on circulating granulocytes was determined by FACS analysis. Heparinized blood samples were placed on ice immediately after blood drawing. After lysis of erythrocytes in isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) for 10 minutes, samples were centrifuged at 400 x g for 5 minutes. The
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cell pellet was washed and kept in PBS containing 0.5% BSA, 1.5 mM sodium azide and 0.35 mM EDTA, at a final concentration of 5 x 10⁶ cells/ml. All procedures were performed at 4°C. The following antibodies were used: CXCR1-fluorescein isothiocyanate (CXCR1-FITC) or CXCR2-phycoerythrin (CXCR2-PE; both antibodies from R&D Systems, Abingdon, UK). All FACS reagents were used in concentrations recommended by the manufacturer. To correct for non-specific staining, all analyses were also conducted with the appropriate control antibodies (FITC and PE labeled murine IgG1 (CLB, Amsterdam, the Netherlands)). At least 10,000 granulocytes were counted in each assay. Mean cell fluorescence (MCF) of forward and side angle scatter-gated granulocytes was assessed using a FACS scan flow cytometer (Becton Dickinson, Mountain View, CA). Data are presented as the difference between MCF intensities of specifically and non-specifically stained cells.

Statistical analysis
All values are given as means ±SEM. Differences in results between the 3 treatment groups were tested by repeated measurements analysis of variance. A value of $P < 0.05$ was considered to represent a statistically significant difference.

Results

Effect of p38 MAPK inhibition on CXC receptor expression
LPS injection induced an initial neutropenia, reaching a nadir at 1 hour after LPS infusion, followed by neutrophilia peaking at 8 hours, as described previously (10). Table I shows total leukocyte and neutrophil counts at the time points at which CXCR expression was assessed by FACS analysis. Inhibition of p38 MAPK with a high (600 mg), but not low (50 mg) dose of BIRB 796 BS attenuated the neutrophilic leukocytosis induced by LPS ($P = 0.001$ vs. placebo, table I). Administration of LPS elicited a transient downregulation of CXCR1 and CXCR2 on circulating neutrophils, confirming our earlier study (2). Neutrophil surface expression of CXCR1 diminished from a baseline (at 0 hour) MCF level of 669.1 (± 93.4) to a nadir of 383.7 (± 31.4) at 2 hours post LPS infusion in the absence of p38 inhibition (Figure 1A). Inhibition of p38 MAPK with a high, but not low dose of BIRB 796 BS attenuated CXCR1 downmodulation (564.4 (± 57.5) at 2 hours vs. 656.9 (± 55.9) at baseline, $P = 0.083$ vs. placebo, Figure 1A). Similarly, CXCR2 neutrophil surface expression was downmodulated during endotoxemia. CXCR2 MCF on neutrophils decreased from 708.5 (± 102.3) at baseline, to a nadir of 127.0 (± 13.1) at 2 hours after LPS infusion in the volunteers
that did not receive BIRB 796 BS (Figure 1B). Neutrophils from volunteers treated with the high dose of p38 MAPK inhibitor displayed significantly less CXCR2 downmodulation during endotoxemia (369.3 (± 42.7) at 2 hours vs. 602.2 (± 86.7) at baseline, \( P = 0.009 \) vs. placebo, Figure 1B). In volunteers receiving a low dose of p38 MAPK inhibitor, CXCR1 and CXCR2 downmodulation was not significantly different from placebo treated volunteers.

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Table 1. Effect of p38 MAPK inhibition on peripheral blood leukocyte and neutrophil counts during human endotoxemia. Subjects received an intravenous injection of LPS (4 ng/kg) at \( t = 0 \) h preceded by oral ingestion of placebo, p38 MAPK inhibitor in a low dose (50 mg BIRB 796 BS) or high dose (600 mg BIRB 796 BS) at \( t = -3 \) h. High dose p38 MAPK inhibitor attenuated the LPS induced initial neutropenia and subsequent neutrophilia (\( P = 0.001 \) vs. placebo).

Figure 1. Effect of p38 MAPK inhibition on CXC receptor expression during human endotoxemia. Subjects received an intravenous injection of LPS (4 ng/kg) at \( t = 0 \) h preceded by oral ingestion of placebo (depicted as □ in the figure), p38 MAPK inhibitor in a low dose (50 mg BIRB 796 BS; ▲) or high dose (600 mg BIRB 796 BS; ○) at \( t = -3 \) h. Neutrophil (A) CXCR1 and (B) CXCR2 surface expression was determined by FACS analysis as described in the methods. Inhibition of p38 MAPK, using 600 mg BIRB 796, attenuated LPS induced CXCR1 (\( P = 0.683 \) vs. placebo) and CXCR2 (\( P = 0.009 \) vs. placebo) downmodulation.
Effect of p38 MAPK inhibition on IL-8 and GRO-α release.

In vitro, IL-8 release from granulocytes and monocytes is dependent on functional p38 MAPK (8). However, to date no reports are available on the in vivo role of p38 MAPK in chemokine production. Infusion of LPS induced a transient rise in the plasma concentrations of IL-8 and GRO-α. Plasma from volunteers treated with either low or high dose p38 MAPK inhibitor displayed significantly reduced levels of IL-8 (placebo: 2457.7 (± 1504.9) pg/ml at 2 hours; 50 mg BIRB 796 BS: 591.3 (± 84.4) pg/ml; 600mg BIRB 796 BS: 310.4 (± 86.5) pg/ml; *P* = 0.013 and *P* = 0.002 for high and low dose respectively vs. placebo, Figure 2A). LPS-induced GRO-α release was similarly reduced in a dose-dependent fashion upon inhibition of p38 MAPK (placebo: 512.0 (± 96.5) pg/ml at 3 hours; 50 mg BIRB 796 BS: 241.0 (± 85.3) pg/ml; 600mg BIRB 796 BS: 101.2 (± 15.3) pg/ml, *P* < 0.001 vs. placebo for both active dose levels, Figure 2B).

Figure 2. Effect of p38 MAPK inhibition on IL-8 and GRO-α release during human endotoxemia. Subjects received an intravenous injection of LPS (4 ng/kg) at t = 0 h preceded by oral ingestion of placebo (depicted as ◦ in the figure), p38 MAPK inhibitor in a low dose (50 mg BIRB 796 BS; ▲) or high dose (600 mg BIRB 796 BS; ◆) at t = -3 h. (A) Inhibition of p38 MAPK, using 50 mg or 600 mg BIRB 796, reduced IL-8 plasma levels during human endotoxemia (*P* = 0.013 vs. placebo and *P* = 0.002 vs. placebo, respectively). (B) Inhibition of p38 MAPK, using 50 mg or 600 mg BIRB 796, reduced GRO-α plasma levels during human endotoxemia (for both doses: *P* < 0.001 vs. placebo).

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Discussion

MAPKs are signal transduction proteins involved in numerous inflammatory events. In vitro, p38 MAPK is activated in neutrophils upon stimulation with LPS and GRO-α, and neutrophil effector functions such as respiratory burst and chemotaxis depend on functional p38 MAPK (6). We recently demonstrated that inhibition of p38 MAPK markedly reduces markers of neutrophil activation such as elastase, up-regulation of CD11b, and down-modulation of L-selectin (10). Chemokine receptors CXCR1 and CXCR2 and their ligands IL-8 and GRO-α are critical determinants of neutrophil migration, and insight into their regulation enhances our understanding of the pathogenesis of many infectious and inflammatory diseases (1). However, to date little is known about the factors that regulate expression of CXC receptors and chemokine production in vivo. In the present study we extend our knowledge to describe a role for p38 MAPK in regulating IL-8 and GRO-α and their receptors during systemic inflammation in humans.

LPS infusion downmodulated CXCR1 and CXCR2 expression to a minimum of 57% and 20% respectively from baseline expression, confirming our earlier observations (2). Inhibition of p38 MAPK resulted in attenuation of receptor downmodulation, although this effect was only apparent in the high dose BIRB 796 BS-treated volunteers. Downmodulation of chemokine receptor after LPS infusion is thought to reflect a protective mechanism that shields the body from harmful effects of sustained infiltration and prolonged activation of neutrophils, and is thought to involve metalloproteinase-mediated cleavage of the receptor (12). Interestingly, involvement of p38 MAPK in metalloproteinase expression was demonstrated recently (13), and thus it is very well conceivable that CXCR downmodulation
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is mediated through a p38 MAPK-regulated metalloproteinase-dependent pathway. In vitro, LPS-induced CXCR1 and CXCR2 downmodulation is independent of TNFα (12). As BIRB 796 BS dose-dependently inhibited TNFα release (10), but not CXCR1 and CXCR2, our study seems to corroborate these findings in vivo.

In vitro, p38 MAPK is involved in IL-8 production in neutrophils (8). A direct role of p38 MAPK in production of GRO-α has not been demonstrated. Here we report that the transient increase of plasma IL-8 and GRO-α levels observed during human endotoxemia, is dose-dependently diminished by a specific p38 MAPK inhibitor. Plasma levels of GRO-α peaked later than those of IL-8. Recently, Fujisawa et al., showed that injection of IL-8 into a rabbit knee joint induced GRO-α release (and vice-versa) (14). We cannot exclude the possibility that LPS-induced GRO-α production is an indirect result of elevated levels of IL-8, and that the observed inhibitory effect of BIRB 796 on GRO-α release is a consequence of a p38 MAPK inhibitor-induced decrease in IL-8 levels. Similarly, the strong reduction in LPS-induced TNFα release in subjects receiving BIRB 796 BS (10) may have contributed to the attenuated secretion of IL-8, considering that IL-8 release is largely TNFα dependent in this model of low-grade endotoxemia (15). The nadir in CXCR expression coincided with peak IL-8 concentrations. Although IL-8 can reduce the expression of both CXCR1 and CXCR2 (3, 16, 17), we consider a direct effect of LPS, at least in part mediated via p38 MAPK, more likely to be responsible for the diminished CXCR expression. Indeed, only very high IL-8 levels (several logs higher than achieved in vivo here) can modulate CXCR expression in vitro and we previously showed that LPS induced a very fast and strong downregulation of CXCR expression in vitro (i.e. within one hour and thus long before IL-8 release can be detected), which in part could be reversed by inhibition of p38 MAPK (2).

It should be noted that the model used only allows for examination of neutrophils that remain within the circulation after LPS injection. Therefore, CXCR expression was only analyzed on this selected cell population. Alterations in CXCR expression on cells present in tissues (like alterations in tissue IL-8 concentrations) cannot be evaluated in this model in healthy human volunteers because of ethical reasons.

Our data show that a p38 MAPK inhibitor is able to modulate neutrophil chemokine receptors CXCR1 and CXCR2 and their ligands IL-8 and GRO-α during inflammation in humans.
Inhibition of p38 MAPK may play a role in modulating neutrophil migration in inflammatory diseases in humans.

References


