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P38 MAPK is involved in the downregulation of granulocyte CXCR 1 and 2 during human endotoxemia

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Abstract

Chemokine receptors CXC receptor 1 and 2, and their ligands interleukin-8 and growth related oncogene-α are principal regulators of neutrophil activation and migration. In vitro, p38 mitogen activated protein kinase plays an important role in regulating these migratory factors, however, little is known about their regulation in vivo. We employed the human endotoxemia model to investigate the role of p38 mitogen activated protein kinase in modulating CXC receptor 1 and 2 expression and release of interleukin-8 and growth related oncogene-α during systemic inflammation in vivo. 24 healthy volunteers received a specific p38 mitogen activated protein kinase-inhibitor in either a high dose, a low dose, or placebo. Subsequently lipopolysaccharide was infused. Endotoxemia-induced reduction of neutrophil CXC receptor 1 and 2 expression, as determined by fluorescence-activated cell sorter analysis, was significantly inhibited in volunteers receiving a high dose of the p38 mitogen activated protein kinase-inhibitor. Endotoxemia-induced rise in plasma interleukin-8 and growth related oncogene-α levels was dose-dependently diminished by the kinase inhibitor. These results indicate a principal role for p38 mitogen activated protein kinase in regulating essential factors for neutrophil activation and chemotaxis in vivo.
MAPK and chemokines in vivo

Introduction

Neutrophil migration is a hallmark of many acute and chronic inflammatory diseases. During infectious disease, influx of neutrophils and subsequent production of reactive oxygen species and proteolytic enzymes form an important line of defense in the innate immune response to invading microorganisms\(^{50,51}\). On the other hand, during chronic inflammatory diseases neutrophil products can have a destructive effect on e.g. joint and gut tissue\(^{137,138}\). In these instances, interference in neutrophil migration seems an attractive target in combating disease, and inhibition of chemotaxis has been suggested as a new therapy for rheumatoid arthritis, Crohn's disease, and asthma\(^{139-141}\).

Granulocyte activation and migration to the site of inflammation is regulated by CXC chemokines, a family of small proteins with strong chemotactic activity. Members of the CXC chemokine family that stimulate granulocyte functions include interleukin (IL)-8; growth-related oncogenes (GRO)\(\alpha\), GRO\(\beta\), and GRO\(\gamma\); and epithelial-derived neutrophil attractant (ENA)78\(^{50,51}\). Granulocytes express 2 types of CXC chemokine receptors (CXCR) that interact with these mediators: CXCR1, which exclusively binds IL-8, and CXCR2, which, besides IL-8, can also bind GROs and ENA-78\(^{50,51}\).

In general, granulocytes seem to respond to infectious and inflammatory agents with a downregulation of CXCR1 and -2 at their surface. Indeed, in vitro CXCR1 and CXCR2 are down-regulated upon stimulation with IL-8, CXCR2 expression can also be diminished by GRO\(\alpha\), lipopolysaccharide (LPS), Tumor Necrosis Factor (TNF)\(\alpha\), and fMLP\(^{142-146}\). In vivo, CXCR1 and CXCR2 expression on granulocytes is reduced during chronic lower respiratory tract infection, lung tuberculosis, human immunodeficiency virus (HIV) infection, and experimental human endotoxemia\(^{52,147,148}\), whereas during sepsis only granulocyte CXCR2 expression is decreased\(^{149}\). Interestingly, however, little is known about the intracellular mechanisms regulating 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\(^{52}\). MAPKs are important intracellular transducers of inflammatory signals. Especially p38 MAPK plays a cardinal role in regulating stress-induced events, such as cytokine production,
during inflammation. P38 MAPK is activated upon stimulation of neutrophils with LPS, and inhibition of p38 MAPK diminishes IL-8 production in monocyte, granulocytes and endothelial cells. Furthermore, OxLDL induced CXCR2 expression on monocytes was shown to be dependent on functional p38 MAPK in vitro. Thus, in vitro evidence supports a role for p38 MAPK in regulating CXC receptor and ligand expression.

In vivo relatively little is known about the role of p38 MAPK in regulating CXCR expression. In the present study we extend our previously reported in vitro data and show an important role for p38 MAPK in regulating granulocyte CXCR1 and CXCR2 and their ligands GROα and IL-8 during systemic inflammation in vivo.

Methods

LPS administration to humans in vivo

The data in this study were generated simultaneously with a previously reported study. The study was approved by the institutional scientific and ethics committees, and written informed consent was obtained from each subject prior to the start of the study. Twenty four healthy male volunteers (mean age 22, range 19-29 years) participated in the investigation. A new, highly specific, orally ingested p38 MAPK inhibitor was used: BIRB 796 BS (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT). Eight subjects received 600 mg BIRB 796 BS (high dose), eight subjects received 50 mg BIRB 796 BS (low dose) and eight subjects received placebo orally, 3 hours prior to infusion of LPS. LPS (Escherichia coli lipopolysaccharide, lot G1, United States Pharmacopeial Convention, Rockville, MD) was administered as a bolus intravenous injection at a dose of 4 ng/kg body weight as described earlier. Blood was obtained from an intravenous canula before administration of BIRB 796 BS or placebo (t = -3 hours), directly before LPS administration (t = 0 hour), and at 5, 15, 30, 60 and 90 minutes and 2, 3, 4, 5, 6, 8, 10 and 24 hours thereafter.
Cytokine measurements

IL-8 and GROα 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; GROα: R&D Systems, Abingdon, UK).

FACS analysis

Expression of CXCR1 and CXCR2 on circulating granulocytes was determined in heparinized blood obtained at -3, 0, 2, 4, 6, 8, 10 and 24 hours relative to LPS injection. All 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 remaining cells were washed and subsequently 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.
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**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 hours 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 hours. Neutrophil CXCR1 and 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 = 0.083 vs. placebo) and CXCR2 (p = 0.009 vs. placebo) downmodulation.

**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. Administration of LPS elicited a transient downregulation of CXCR1 and CXCR2 on circulating neutrophils, confirming our earlier study. 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. Inhibition of p38 MAPK, using a high dose of BIRB 796, 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. Baseline CXCR2 MCF on neutrophils decreased from 708.5 (±95.7) at baseline, to a nadir of 127.0 (±12.2) at 2 hours after LPS infusion. Neutrophils from volunteers treated with the high dose of p38 MAPK inhibitor displayed significantly less CXCR2 downmodulation during endotoxemia (427.67 (±67.1) at 2 hours vs. 602.23 (±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.
Figure 2: Effect of p38 MAPK inhibition on IL-8 and GROα release during human endotoxemia. Subjects received an iv injection of LPS (4 ng/kg) at t = 0 hours 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 hours. (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, p = 0.002 vs. placebo, respectively). (B) Inhibition of p38 MAPK, using 50 mg or 600 mg BIRB 796, reduced GROα plasma levels during human endotoxemia (for both doses: p <0.001 vs. placebo).

Effect of p38 MAPK inhibition on IL-8 and GROα release.

In vitro, IL-8 release from granulocytes and monocytes is dependent on functional p38 MAPK. 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 GROα, corroborating earlier reports.

Plasma from volunteers treated with either low (50 mg BIRB 796) or high dose (600 mg BIRB 796) p38 MAPK inhibitor displayed significantly reduced levels of IL-8 (Placebo: 2457.7 (± 1504.9) pg/ml at 2 hours; 50 mg BIRB 796: 591.3 (± 84.4) pg/ml; 600mg BIRB 796: 310.4 (± 86.5) pg/ml, p = 0.013, p = 0.002 respectively vs. placebo, Figure 2A). Similarly, LPS-induced GROα release was 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: 241.0 (± 85.3) pg/ml; 600mg BIRB 796: 101.2 (± 15.3) pg/ml, both p <0.001 vs. placebo, Figure 2B). Notably, the rise in GROα levels was relatively slow compared to IL-8: GROα levels started rising at 1 hour (vs. IL-8 at 30 minutes), and peaked not until 3 hours after LPS infusion (vs. IL-8 at 2 hours).
**Discussion**

MAPKs are signal transduction proteins involved in numerous inflammatory events. In vitro, p38 MAPK is activated in neutrophils upon stimulation with LPS and GROα, and neutrophil effector functions such as respiratory burst and chemotaxis depend on functional p38 MAPK\(^{94,110,155,156}\). Furthermore, we recently showed that an inhibitor of p38 MAPK potently reduced neutrophil activation markers such as elastase, up-regulation of CD11b, and down-modulation of L-selectin\(^{152}\). Chemokine receptors CXCR1 and CXCR2 and their ligands IL-8 and GROα are critical determinants of neutrophil migration, and insight into their regulation enhances our understanding of the pathogenesis of many infectious and inflammatory diseases\(^{50,51,153}\). 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 describe an important role for p38 MAPK in regulating IL-8 and GROα and their receptors during systemic inflammation in humans.

LPS infusion downmodulated CXCR1 and CXCR2 expression up to 57% and 20%, respectively, of baseline expression, confirming our earlier observations\(^{52}\). Inhibition of p38 MAPK resulted in attenuation of receptor downmodulation, although this effect was only apparent in the high dose BIRB 796-treated volunteers. LPS induced chemokine receptor downmodulation 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\(^{143}\). Interestingly, involvement of p38 MAPK in metalloproteinase expression was recently demonstrated\(^{157,158}\), and thus it is very well conceivable that CXCR downmodulation is mediated through a p38 MAPK-regulated metalloproteinase-dependent pathway. In vitro, LPS-induced CXCR1 and CXCR2 downmodulation is independent of TNFα\(^{143}\). As BIRB 796 dose-dependently inhibited TNFα release\(^{152}\), 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\(^{53,94}\). Interestingly, it is unknown whether p38 MAPK is directly involved in production of GROα. Here we report that the transient increase of plasma IL-8 and GROα levels, as is observed during human endotoxemia, is dose-dependently diminished by a specific p38 MAPK inhibitor.
Interestingly, plasma levels of GROα peaked relatively late compared with IL-8. Recently, Fujiwara et al., showed that injection of IL-8 into a rabbit knee joint induced GROα release (and vice-versa)\textsuperscript{159}. We cannot exclude the possibility that LPS-induced GROα production is an indirect result of elevated levels of IL-8, and that the observed inhibitory effect of BIRB 796 on GROα 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\textsuperscript{152} 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\textsuperscript{41,126}.

Our data show that a p38 MAPK inhibitor is a potent modulator of neutrophil chemokine receptors CXCR1 and CXCR2 and their ligands IL-8 and GROα during inflammation in humans. Recently, leukocyte trafficking has been suggested as a novel therapeutic target for inflammatory diseases\textsuperscript{139-141}. In this respect, it should prove interesting to see whether inhibition of p38 MAPK might play a role in modulating neutrophil migration in inflammatory diseases in humans.

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