In vivo signal transduction: mitogen activated protein kinases in inflammation
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chapter

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Anti-inflammatory effects of a p38 Mitogen Activated Protein Kinase inhibitor during human endotoxemia


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Abstract

p38 MAPK participates in intracellular signaling cascades resulting in inflammatory responses. Therefore, inhibition of the p38 MAPK pathway may form the basis of a new strategy for treatment of inflammatory diseases. However, p38 MAPK activation during systemic inflammation in humans has not yet been shown, and its functional significance in vivo remains unclear. Hence, we exposed 24 healthy male subjects to an intravenous dose of LPS (4 ng/kg) preceded 3 hours earlier by orally administered 600 or 50 mg BIRB 796 BS (an \textit{in vitro} p38 MAPK inhibitor), or placebo. Both doses of BIRB 796 BS significantly inhibited LPS-induced p38 MAPK activation in the leukocyte fraction of the volunteers. Cytokine production (TNF\(\alpha\), IL-6, IL-10, and IL-1 receptor antagonist) was strongly inhibited by both low and high dose p38 MAPK inhibitor. In addition, p38 MAPK inhibition diminished leukocyte responses including neutrophilia, release of elastase-\(\alpha\)-1-antitrypsin complexes and upregulation of CD11b with downregulation of L-selectin. Finally, blocking p38 MAPK decreased C-reactive protein release. These data identify p38 MAPK as a principal mediator of the inflammatory response to LPS in humans. Furthermore, the anti-inflammatory potential of an oral p38 MAPK inhibitor in humans \textit{in vivo} suggests that p38 MAPK inhibitors may provide a new therapeutic option in the treatment of inflammatory diseases.
Introduction

Diseases such as rheumatoid arthritis and Crohn's disease are characterized by chronic inflammation leading to destruction of normal tissue integrity. Mediators released during inflammatory diseases activate intracellular signaling cascades regulated by kinase and phosphatase enzymes. The MAPKs are part of such signaling cascades at which diverse extracellular stimuli converge to initiate inflammatory cellular responses. Several subgroups have been identified within the MAPK family, including the p42/44 extracellular signal-related kinases, JNK and p38 MAPks. p38 MAPK has been implicated as an important regulator of the co-ordinated release of cytokines by immunocompetent cells and the functional response of neutrophils to inflammatory stimuli. Many different stimuli can activate p38 MAPK. These include LPS and other bacterial products, cytokines such as TNFα and IL-1, growth factors and stresses such as heat shock, hypoxia and ischaemia/reperfusion. In addition, p38 MAPK positively regulates a variety of genes involved in inflammation such as TNFα, IL-1, IL-6, IL-8, cyclooxygenase-2, and collagenases-1 and -3.

Because of the broad pro-inflammatory role of p38 MAPK in several in vitro systems, inhibition of this pathway has been advocated as a novel therapeutic strategy for inflammatory diseases. However, the effect of p38 MAPK inhibition in in vivo models of inflammation has only been examined in a limited number of studies with equivocal results. p38 MAPK inhibitors have been found to reduce LPS-induced TNFα production in mice and rats. This result could not be duplicated with one of these inhibitors (SB203580) in mice despite almost completely abolishing the p38 MAPK activity in spleen cells harvested from these animals. Furthermore, inhibition of p38 MAPK was associated with a decrease in neutrophil recruitment and TNFα release in bronchoalveolar lavage fluid in mice after intratracheal administration of LPS, but with elevated TNFα concentrations in lungs during murine pneumococcal pneumonia and tuberculosis. In a murine model of peritonitis induced by cecal ligation and puncture, delayed administration of SB203580 improved survival, and prevented the enhanced release of IL-10 by macrophages harvested from mice with peritonitis, while concurrently improving the reduced IL-12 release by these cells.
Knowledge of the activation of p38 MAP and its role in inflammation in humans in vivo is limited despite current interest in p38 MAPK inhibition in the treatment of human inflammatory disease. In addition, there are conflicting animal data. Therefore, in the present study we used the well characterized model of human inflammation produced by intravenous injection of low dose LPS, to evaluate the activation of p38 MAPK and the effect of a new orally administered p38 MAPK inhibitor.

Methods

p38 MAPK inhibitor

The p38 MAPK inhibitor used in this study (BIRB 796 BS) was developed by Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, CT. BIRB 796 BS is 1-(5-tert-Butyl-2-p-tolyl-2H-pyrazol-3-yl)-3-(4-(2-moprolin-4-yl-ethoxy)-naphtalen-1-yl)-urea (empirical formula C_{31}H_{37}N_{5}O_{3}; molecular weight 527.6), a water soluble, orally bioavailable molecule. Details about the structure and specificity of BIRB 796 BS for p38 MAPK will be published in a separate manuscript. BIRB 796 BS has a greater than 330-fold selectivity for p38 MAPK compared to 12 other protein kinases studied. In contrast to other p38 MAPK inhibitors (e.g. SB203580), BIRB 796 BS prevents both phosphorylation and kinetic activity of p38 MAPK by binding to a novel allosteric binding site as well as to the ATP pocket of p38 MAPK.

Effect of BIRB 796 BS on p38 MAPK activation in vitro

Blood from healthy volunteers was collected with 10 U/ml heparin (Leo Pharmaceutical Products, Weesp, the Netherlands). PBMCs were isolated by density-gradient sedimentation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) from blood diluted 1:1 with PBS. PBMCs were washed twice with PBS and resuspended in RPMI supplemented with 10% heat inactivated FCS (both GIBCO, Grand Island, NY) at a concentration of approximately 5 x 10^6 cells/ml in 15 ml tubes (Becton Dickinson, Franklin Lakes, NJ). After pre-incubation for 1 h with BIRB 796 BS (1, 10, 100 or 1000 nM) or DMSO as the solvent control, samples (containing approximately 5 x 10^6 cells each) were stimulated with LPS from E. coli serotype 0111:B4 (25 ng/ml; Sigma, St. Louis, MO). After 15 min, 12 ml of ice-cold PBS was added
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to each 15 ml tube, and cells were centrifuged at 400 g for 5 min at 4 °C. The cell pellets were lysed in 100 µl 3x SDS-sample buffer; this mixture was briefly sonicated (2 x 10 s) and boiled for 5 min followed by brief centrifugation and storage at -20 °C, until further analysis. As for equal loading during Western blotting (see further), equal amounts of sample (25µl; containing approximately 1.25 x 10^6 cells) were analyzed. Furthermore, after immunoblotting the blots were also subjected to Amido Black staining to assess equal loading.

**Western blotting**

Samples mixed with SDS-sample buffer were loaded on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Subsequently, membranes were blocked in 5% BSA in PBS supplemented with 0.1% Tween-20 and washed in 0.2% BSA in PBS supplemented with 0.1% Tween-20. The extent of p38 MAPK activation was determined using antibodies against phosphorylated (Thr^{180}/Tyr^{182}) p38 MAPK (New England Biolabs, Beverly, MA), used at a 1:1000 dilution incubated overnight. After three washes for 10 min, secondary antibody incubation was performed for 1 hour with Horseradish Peroxidase-conjugated goat-anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark) at a 1:2000 dilution in 10% human serum in PBS supplemented with 0.1% Tween-20. After enhanced chemoluminescence using Lumilight® substrate, antibody binding was visualized using a Lumimage (Boehringer Mannheim, Mannheim, Germany).

**LPS administration to humans** in vivo

The study was performed as a randomized, double-blind, placebo-controlled experiment. 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. All subjects were in good health, as documented by history, physical examination, electrocardiogram, and routine laboratory screening. Tests for HIV, hepatitis B and C were negative. The participants did not use any medication. All participants were non-smokers. The subjects fasted overnight before LPS administration. On the study day, two intravenous canulas were inserted, one for LPS administration and one for blood collection. 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. BIRB 796 BS was given as an oral solution in 15 ml PEG 400. The placebo solution consisted of 15 ml of PEG 400. The study drug and placebo were administered 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. Oral temperature, blood pressure and heart rate were measured every 30 minutes during the first 2 hours after LPS challenge, thereafter at 1 hourly intervals for four hours, then at a decreased frequency. Clinical symptoms such as headache, chills, myalgia, nausea, vomiting, abdominal pain and backache were recorded throughout the study using a graded scale (0 as absent, 1 as mild, 2 as moderate and 3 as severe). Blood was obtained from an intravenous canula before administration of BIRB 796 BS or placebo (t = -3 h), directly before LPS administration (t = 0 h), and at 5, 15, 30, 60 and 90 minutes and 2, 3, 4, 5, 6, 8, 10 and 24 hours thereafter.

**Measurement of BIRB 796 BS plasma levels**

Plasma samples were analyzed for BIRB 796 BS concentrations using a validated high performance liquid chromatography (HPLC) method with electrospray ionization MS/MS (mass spectrometry) detection. Following the solid phase extraction of the analyte from plasma, BIRB 796 BS and the internal standard (d8-BIRB 796 BS) were separated chromatographically followed by detection via the Tandem mass spectrometer. The linear range was established with calibration standards from 0.1 to 1000 ng/ml using peak height ratios. The lower limit of quantitation was 0.1 ng/ml using 500 ml of plasma.

**p38 MAPK measurements during the in vivo study**

Blood for measurement of p38 MAPK was collected in heparin-containing vacutainer tubes at t = 0, 5, 15, 30 and 60 min, and at 4, 8 and 24 h relative to LPS injection. After collection, erythrocytes from 4.5 ml aliquots were lysed by adding 40 ml ice-cold isotonic NH₄Cl solution (155 mmol/l NH₄Cl, 10 mmol/l KHCO₃, 0.1 mmol/l EDTA, pH 7.4, and 1 mM Pefabloc) for 30 min. The remaining leukocytes were centrifuged for 5 min, 400 g, at 4 °C, washed twice with ice cold PBS, and resuspended in 400 µl of PBS. Two hundred µl of the
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cell suspension were added to 125 μl of 3x SDS-sample buffer, this mixture was briefly sonicated (2 x 10 s) and boiled for 5 min followed by brief centrifugation and storage at -20 °C. To the remaining cell suspension, intended for the kinase assay, 800 μl ice-cold cell lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-Glycerolphosphate, 1 mM Na₃VO₄, 1 μg/ml Leupeptin and 1mM Pefabloc) were added. Samples were sonicated 4 x 5 s on ice and centrifuged at 7000 g for 10 min at 4 °C. Protein content in the clear supernatant was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL), using BSA as the standard, and the supernatant was stored at -80 °C.

For all subjects phosphorylation of p38 MAPK was measured by Western blot. Antibody binding was quantified using image analysis software (EFM Software, Rotterdam, the Netherlands) and measurements were corrected for the amount of protein loaded to allow quantitative comparison of p38 phosphorylation between blood samples, which may contain different amounts of leukocyte protein as a consequence of the LPS challenge. The sample obtained at t = -3 h was not adequately treated and was omitted from the analysis. All other samples were compared to one random sample set at an arbitrary value of 100 units, for comparing p38 MAPK phosphorylation between subjects. In addition, a minimum measured activity was considered to be necessary for use as an internal reference (i.e. 25% on a 2⁸ digital gray scale). Two subjects failed to meet this requirement (one from the low dose and one from the high dose BIRB 796 BS treated volunteers), possibly due to low protein yield in these samples, and were not included in the analysis.

p38 MAPK enzymatic activity was measured using a kinase assay (New England Biolabs, Beverly, MA). White blood cell lysates were prepared as described above. A once diluted slurry of agarose hydrazide-bound antibodies to phosphorylated (Thr¹⁸⁰/Tyr¹⁸²) p38 MAPK (40 μl) was utilized to selectively immunoprecipitate active p38 MAPK from the cell lysate by gently shaking overnight at 4 °C. To assure equal loading, a fixed amount of lysate was used per sample (approximately 80 μg in 340 μl cell lysis buffer). The immunoprecipitate was washed twice with 500 μl of ice cold cell lysis buffer and twice with 500 μl of ice cold kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na₃VO₄, 10 mM MgCl₂) at 4 °C. The kinase reactions were carried out in the presence of 200 μM ATP and 2 μg of ATF-2 fusion protein at 30 °C for 30 min. After the reaction had been
terminated by the addition of 3x SDS-sample buffer, the mixture was boiled for 5 min followed by brief centrifugation. ATF-2 phosphorylation was selectively measured by Western immunoblotting as described previously using specific antibodies against phosphorylated (Thr$^{71}$) ATF-2.

Other assays

Cytokine concentrations were determined in EDTA-anticoagulated plasma by specific ELISA’s according to the manufacturers’ instructions (with detection limits). These ELISA’s were: TNFα (2.8 pg/ml), IL-6 (1.2 pg/ml), IL-10 (2.4 pg/ml) (all Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, the Netherlands), and IL-1 receptor antagonist (IL-1ra; 410 pg/ml; R&D Systems, Minneapolis, MN). Elastase-α$_1$-antitrypsin complexes in EDTA plasma were measured with an ELISA modified from a previously described radio-immunoassay procedure$^{119}$. Briefly, ELISA plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with polyclonal rabbit antibodies against human elastase, and incubated with the samples to be tested. Bound complexes were detected by incubation with biotinylated monoclonal antibodies against complexed α$_1$-antitrypsin and streptavidin-peroxidase. Results were referred to a standard curve consisting of pooled human plasma supplemented with purified elastase, and expressed as ng of elastase per ml. CRP was measured in serum by ELISA (detection limit 3 mg/L) according to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany).

FACS analysis

Leukocyte counts and differentials were assessed in EDTA-anticoagulated blood using a Stekker analyzer (counter STKS, Coulter counter, Bedfordshire, United Kingdom). Expression of CD11b (Mac-1) and L-selectin (CD62L) on circulating granulocytes was determined in heparinized blood obtained at -3, 0, 2, 4, 6 and 24 h relative to LPS injection. All blood samples were placed on ice immediately after blood drawing. After lysis of erythrocytes in isotonic NH$_4$Cl solution (155 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA, pH 7.4) for 10 minutes, samples were centrifuged at 400 g for 5 min. 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: FITC labeled mouse anti-human L-selectin (Immunotech, Marseilles, France) and PE labeled mouse anti-human CD11b (Immunotech, Marseilles, France). 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. MCF of forward and side angle scatter-gated granulocytes was assessed using a FACS scan flow cytometer (Beeton Dickinson, Mountain View, CA). Data are presented as the difference between MCF intensities of specifically and non-specifically stained cells.

Statistical analysis

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

Figure 1: Effect of BIRB 796 on LPS induced p38 MAPK phosphorylation in PBMC’s. PBMC’s were stimulated with LPS (+LPS) or solvent control (Co) in the presence of various concentrations BIRB 796. p38 MAPK activation was measured using Western blotting and antibodies against phosphorylated p38 MAPK (pp38). Results were analyzed using image analysis software and are represented as bars (arbitrary units) A representative experiment (out of 4) is shown.
Chapter 3

Results

*BIRB 796 BS is a potent p38 MAPK inhibitor in vitro*

In order to assess the effect of BIRB 796 BS on p38 MAPK activity, we stimulated PBMCs with LPS in the presence of increasing concentrations of BIRB 796 BS or diluent (Figure 1). BIRB 796 BS inhibited LPS-induced phosphorylation of p38 MAPK in a dose-related fashion.

**Figure 2: BIRB 796 BS inhibits LPS-induced p38 MAPK activation in vivo.** 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), 50 mg BIRB 796 BS (○) or 600 mg BIRB 796 BS (▲). Venous blood was obtained at indicated time points, erythrocytes were lysed and the total white blood cell fraction was lysed and assayed for p38 MAPK phosphorylation and enzymatic activity. (A) p38 MAPK phosphorylation in leukocytes. Results (mean ± SEM) were obtained by Western blotting with phosphospecific antibodies and expressed relative to a random sample, which was set on a 100 arbitrary units. (B) Inhibition of p38 MAPK enzymatic activity by BIRB 796 BS. p38 MAPK enzymatic activity was determined by measuring phosphorylation of ATF-2 (pATF-2) in an in vitro kinase assay, using immunoprecipitated phosphorylated p38 MAPK from leukocyte lysates. Results were analyzed using image analysis software and are represented as bars (arbitrary units).

*BIRB 796 BS inhibits LPS-induced p38 MAPK activation in vivo*

Plasma levels of BIRB 796 BS determined after oral ingestion of the p38 MAPK inhibitor peaked at 0.5-2 h after LPS injection (low dose group 0.74 ± 0.25 μM; high dose group 7.38 ± 1.64 μM, i.e. within the same range as the concentrations used in the in vitro experiment). Although there is abundant evidence that active p38 MAPK is involved in LPS-induced
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cytokine production in vitro\textsuperscript{48,120,121}, it is unknown whether p38 MAPK has a similar role in humans in vivo. To investigate the activation of p38 MAPK in human endotoxemia and the effectiveness of BIRB 796 BS in inhibition of p38 MAPK phosphorylation, we measured p38 MAPK activation using phosphospecific antibodies at various time points before and after LPS injection in healthy human subjects. As shown in figure 2A, administration of LPS resulted in p38 MAPK activation in subjects who did not receive BIRB 796 BS, peaking at 60 min. Both low dose and high dose BIRB 796 BS significantly inhibited p38 MAPK activation (both $P < 0.05$ vs. placebo). To further establish the effectiveness of BIRB 796 BS in inhibiting p38 MAPK enzymatic activity in vivo, a kinase assay was performed on white blood cell lysates (an example out of three is shown in figure 2B). In subjects who did not receive BIRB 796 BS, enhanced p38 MAPK enzymatic activity was observed 60 min after LPS administration relative to $t = 0$ h. In contrast, treatment with BIRB 796 BS almost completely prevented p38 MAPK activation at 60 min. These results are, to our knowledge, the first demonstration of p38 MAPK activation in a human model of inflammation and show that BIRB 796 effectively inhibits p38 MAPK in vivo.

\textit{Inhibition of p38 MAPK reduces LPS-induced clinical signs and symptoms}

LPS injection induced symptoms, consisting of fever, chills, myalgia, headache, nausea, vomiting, abdominal pain and backache. In subjects treated with LPS and placebo, mean body temperatures peaked after 3 hours (38.3 ± 0.2 °C). Although both doses BIRB 796 BS tended to reduce the febrile response, this effect did not reach statistical significance (Figure 3). BIRB 796 BS treatment did attenuate LPS-induced symptoms, both in incidence and severity, and delayed the time point of maximal presentation (Table I). Apparently activation of p38 MAPK is involved in the generation of the LPS-induced clinical signs and symptoms.
Table I. BIRB 796 BS reduces LPS-induced clinical symptoms

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Placebo (n = 8)</th>
<th>BIRB 796 BS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>S/mo/mi^3</td>
</tr>
<tr>
<td>Headache</td>
<td>8</td>
<td>1/3/4</td>
</tr>
<tr>
<td>Chills</td>
<td>7</td>
<td>0/2/5</td>
</tr>
<tr>
<td>Myalgia</td>
<td>4</td>
<td>0/1/3</td>
</tr>
<tr>
<td>Nausea</td>
<td>5</td>
<td>0/2/3</td>
</tr>
<tr>
<td>Vomiting</td>
<td>3</td>
<td>1/1/1</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>2</td>
<td>0/2/0</td>
</tr>
<tr>
<td>Backache</td>
<td>1</td>
<td>0/0/1</td>
</tr>
</tbody>
</table>

1 Subjects received an i.v. injection of LPS (4 ng/kg) at t = 0 h preceded by oral ingestion of placebo, 50 mg BIRB 796 BS, or 600 mg BIRB 796 BS.
2 Total number of subjects suffering from the indicated symptom.
3 A further distinction was drawn between severe (s), moderate (mo), and mild (mi) symptoms.
4 Peaking time refers to the maximum number of subjects suffering from the indicated symptom.
5 N/A, Not applicable.

Figure 3: BIRB 796 BS tends to reduce the febrile response to LPS. 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), 50 mg BIRB 796 BS (○) or 600 mg BIRB 796 BS (▲) at t = -3 h, and body temperatures (given as mean ± SEM) were measured orally. The difference between treatments was not significant.
p38 MAPK inhibition diminishes LPS-induced cytokine release

p38 MAPK positively regulates a number of cytokine genes \textit{in vitro}^{6,114}. Therefore, the effect of pharmacological p38 MAPK inhibition on cytokine release during endotoxemia in humans was examined. Administration of LPS to subjects not treated with BIRB 796 BS elicited transient rises in the plasma concentrations of TNF$\alpha$, IL-6, IL-10, and IL-1ra (Figure 4). All of these cytokine responses were strongly inhibited by both doses of BIRB 796 BS (all $P < 0.05$ vs. placebo except for IL-1ra at low dose). In addition, the inhibitory effect of BIRB 796 BS appeared dose-dependent although the low dose already diminished cytokine release to a statistically significant extent. Thus p38 MAPK activation is required for cytokine release during human inflammation.

![Figure 4: BIRB 796 BS inhibits LPS-induced cytokine release. Subjects received an intravenous injection of LPS (4 ng/kg) at $t = 0$ h preceded by oral ingestion of placebo (depicted as $\square$ in the figure), 50 mg BIRB 796 BS ($\circ$) or 600 mg BIRB 796 BS ($\blacktriangle$) at $t = -3$ h. Data are mean $\pm$ SEM. Both low and high dose BIRB 796 BS inhibited the release of all mediators shown (all $P < 0.05$ vs. placebo) except for IL-1ra release in the low dose group (nonsignificant vs placebo).]
Inhibition of p38 MAPK reduces LPS-induced neutrophil activation

p38 MAPK phosphorylation results in the activation of several pro-inflammatory neutrophil functions in vitro\(^6,114\). Therefore, the effect of BIRB 796 BS on neutrophil activation induced by LPS in vivo, was assessed by measuring neutrophil counts, release of elastase, and expression of CD11b and L-selectin (Figure 5). LPS injection in subjects treated with placebo was associated with a biphasic change in neutrophil numbers in peripheral blood, characterized by an initial neutropenia with a nadir at 1 h, followed by a neutrophilia peaking at 8 h. LPS administration also induced a transient rise in the plasma concentrations of elastase-\(\alpha_1\)-antitrypsin complexes, reflecting neutrophil degranulation\(^119\), and an upregulation of CD11b at the surface of circulating granulocytes with a concurrent down-modulation of L-selectin, indicative for cellular activation\(^122\). Whereas the lower BIRB 796 BS dose tended to attenuate these LPS-induced neutrophil responses (\(P < 0.05\) for L-selectin; \(P \geq 0.05\) for other
parameters vs. placebo), the higher dose of the p38 MAPK inhibitor resulted in significant reduction of all parameters of neutrophil activation (all $P < 0.05$ vs. placebo), suggesting an essential role for p38 MAPK in mediating leukocyte responses in vivo.

**BIRB 796 BS inhibits CRP release**

To obtain insight into the role of p38 MAPK on more subacute consequences of LPS-induced inflammation, CRP concentrations were measured 3 h before and 24 h after LPS injection (Figure 6). LPS induced a profound increase in the serum levels of this acute phase reactant, which was significantly lower in the group receiving BIRB 796 BS ($P < 0.05$ for both doses vs. placebo).

![Figure 6: BIRB 796 BS inhibits LPS-induced CRP release. Subjects received an intravenous injection of LPS (4 ng/kg) at $t = 0$ h preceded by oral ingestion of placebo, 50 mg BIRB 796 BS or 600 mg BIRB 796 BS at $t = -3$ h. Data are mean ± SEM. Both low and high dose BIRB 796 BS inhibited the release of CRP (all $P < 0.05$ vs. placebo).](image)

**Discussion**

The present investigation examines the activation of p38 MAPK during an *in vivo* inflammatory response in humans, along with the effect of a p38 MAPK inhibitor in altering this response. Intravenous injection of LPS elicited transient activation of p38 MAPK, followed by a characteristic inflammatory response including the release of pro-inflammatory cytokines, a neutrophilic leukocytosis, neutrophil activation and acute phase protein release. Oral ingestion of BIRB 796 BS, a specific p38 MAPK inhibitor, attenuated these responses.
These results not only identify p38 MAPK as a principal regulator of the inflammatory response in humans, but also suggest that p38 MAPK inhibition may have significant potential for treating inflammatory disease.

Intravenous administration of LPS induced activation of p38 MAPK in blood, peaking after one hour, as demonstrated by two different methods, Western blotting with antibodies against phosphorylated (Thr\(^{180}\)/Tyr\(^{182}\)) p38 MAPK, and a kinase assay. Using similar methods, it has been previously demonstrated that there is transient activation of p38 MAPK in splenocytes of mice peaking within 15 minutes of intraperitoneal injection of LPS\(^98\). The later activation of p38 MAPK in the human model in comparison to mouse may be related to the differences in the route of LPS administration, the LPS dose administered, and the cell types analyzed.

p38 MAPK activation was measured in lysates of all white blood cells obtained from whole blood, rather than in isolated cell fractions, since we argued that the isolation procedures might affect the activation status of p38 MAPK. Several papers have described the in vitro effect of LPS on different leukocyte cell fractions without showing apparent effects of the different separation methods on basal p38 MAPK activation. However, in contrast to these in vitro studies, in vivo endotoxemia studies do not allow cell separation before exposure to LPS. Furthermore, in our experience more elaborate sample handling can lead to activation of kinases (data not shown). In order to accurately assess the activation state of p38 MAPK at several time points during human endotoxemia, we therefore felt it was warranted to process the in vivo stimulated whole blood samples quickly and with minimal intervention. Unfortunately, no information concerning the relative contribution of the several leukocyte subsets to the beneficial effect of MAPK inhibition can be gathered in this fashion. In the future intracellular FACS analysis of MAPK activation, a method that currently is evaluated in our laboratory, might solve this problem.

BIRB 796 BS, given orally at either 50 or 600 mg, strongly reduced p38 MAPK activation \textit{in vivo} (Figure 2). Both BIRB 796 BS doses attenuated LPS-induced inflammatory responses, although the higher dose appeared to exert stronger inhibitory effects. Of note, inflammatory effects produced by LPS were not abrogated completely. BIRB 796 BS at the doses used in this study may not completely inhibit p38 MAPK activity, either in the blood, or in immunocompetent cells not present in the circulation (endothelial cells, fibroblasts and tissue
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macrophages). In addition, p38 MAPK independent pathways of inflammation may also contribute to some of the effects seen.

Inhibition of p38 MAPK was associated with a profound reduction in the release of both pro- and anti-inflammatory members of the cytokine network. The strong reduction in LPS-induced TNFα release with the p38 MAPK inhibitor BIRB 796 BS corresponds with the \textit{in vitro} effect of p38 MAPK inhibition in stimulated monocytic cells and neutrophils\textsuperscript{12,14,47,48,123}. In addition, p38 MAPK inhibitors diminished LPS-induced TNFα production in mice and rats \textit{in vivo}\textsuperscript{46,47,116} and mice deficient in MAPKAPK-2, a downstream substrate kinase for p38 MAPK, proved to be resistant to LPS-induced shock\textsuperscript{49}. A previous study in mice was not able to demonstrate an inhibitory effect of the p38 MAPK inhibitor SB203580 on TNFα release after intraperitoneal administration of LPS\textsuperscript{98}. This may be a result not only of species differences, but also of the differences in the mechanism of inhibition of the two drugs. In \textit{vitro} data suggest that besides p38 MAPK other stress signaling pathways, e.g. MAPK family members p42/44 and JNK and the NFκB pathway, are involved in LPS induced TNFα release\textsuperscript{24,30}. Indeed, all three major MAPK family members and NFκB can be activated upon stimulation with LPS\textsuperscript{40,45,124}. Furthermore, the TNFα promotor has binding sites for NFκB as well as for transcription factors under control of the MAPK family (e.g. AP-1)\textsuperscript{40,125}. Thus, full expression of the TNF gene seems to involve activation of several of the before mentioned stress pathways. However, the relative importance of each of these pathways may vary under different conditions. Interestingly, deletion of one of the NFκB binding sites from the TNF promotor had little effect on LPS induced TNFα production\textsuperscript{125}. We observed that inhibition of p38 MAPK decreased endotoxemia induced TNFα plasma levels up to 97%. These data suggest that in the human endotoxemia model the p38 MAPK pathway has little redundancy in respect to TNFα release. The inhibition of cytokine release in subjects treated with BIRB 796 BS could have been related to the reduction in TNFα secondary to p38 MAPK inhibition. Elimination of endogenous TNFα activity with an anti-TNF antibody or a TNF receptor fusion protein in the human LPS model was accompanied by a marked reduction in the release of other cytokines and cytokine inhibitors, including IL-6, IL-10, and IL-1ra\textsuperscript{41-43,126}. In addition, anti-TNF administration resulted in reduced IL-10 release by LPS stimulated monocytes, whereas p38 MAPK inhibition attenuated both TNFα and IL-10 production in this \textit{in vitro} system\textsuperscript{123}. Thus, together with our in vivo findings, these data indicate that p38
MAPK is not only involved in the production of proinflammatory cytokines, but also of anti-inflammatory cytokines. Yet, the subjects treated with BIRB 796 BS demonstrated evidence for an overall anti-inflammatory effect, suggesting that p38 MAPK inhibition predominantly influences proinflammatory pathways.

p38 MAPK is considered important for many different pro-inflammatory neutrophil functions. Although the anti-inflammatory effects of p38 MAPK inhibitors on neutrophils are well known in vitro, little is known about the in vivo relevance of these findings. Inhibition of p38 MAPK has been reported to reduce neutrophil influx into bronchoalveolar lavage fluid after intratracheal administration of LPS in mice. Furthermore, local application of a p38 MAPK inhibitor in the lumen of an ileal loop prior to administration of Clostridium difficile toxin A at the same location, has been associated with a strong reduction in both neutrophil recruitment and the severity of the resulting enteritis in mice. This study demonstrates that p38 MAPK inhibition attenuates the neutrophil response to intravenous LPS in humans in vivo, and reduces the activation of neutrophils as indicated by inhibition of degranulation, the upregulation of CD11b and the downmodulation of L-selectin. These data correspond with previous in vitro reports demonstrating that p38 MAPK inhibition reduces neutrophil degranulation, the shedding of L-selectin and the upregulation of CD11b. BIRB 796 BS also reduced the incidence and severity of clinical symptoms, and delayed the time point of maximal presentation, an effect that likely would have been more clear cut if more subjects would have been studied.

Intravenous injection of LPS induces a reproducible transient inflammatory state in normal subjects that is considered relevant for the investigation of pathophysiologic pathways operative in inflammatory conditions. As such, this model of inflammation in man offers an opportunity to obtain proof of principle for the action of anti-inflammatory compounds. It should be noted that the human endotoxemia model is less suitable to investigate the efficacy of postponed treatment with an anti-inflammatory compound, since the inflammatory response to intravenous LPS is very rapid and transient. Nonetheless, the current findings establish that inhibition of p38 MAPK by the oral administration of BIRB 796 BS exerts anti-inflammatory effects during experimental endotoxemia. These effects are comparable to those seen in this model with anti-TNF antibodies, TNF receptor fusion protein and IL-10 drugs presently used clinically or in trials in the management of chronic
inflammatory diseases such as rheumatoid arthritis and Crohn's disease\textsuperscript{59,134-136}. Clearly, the greatest advantages of compounds like BIRB 796 BS would be their oral availability and lack of immunogenicity in comparison to the biological products. Taken together with (limited) animal data, these results provide hope for the future use of oral p38 MAPK inhibitors in patients with inflammatory diseases.