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Effect of the p38 MAPK inhibitor SB-239063 on Lipopolysaccharide-induced psychomotor retardation and peripheral biomarker alterations in rats

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ABSTRACT

Lipopolysaccharide (LPS) administration in rats induces a characteristic syndrome termed 'sickness behavior', including profound changes on locomotor activity and circulating stress and inflammatory mediators. The aim of the present investigation was to evaluate whether the behavioral and the peripheral biomarker responses induced by LPS could be modified by acute treatment with the p38 mitogen-activated protein kinase inhibitor SB-239063. Male Sprague–Dawley rats were treated orally either with vehicle or SB-239063 (3, 10 and 30 mg/kg) 1 h before an intraperitoneal injection of either saline or LPS 125 μg/kg. Two hours after LPS injection, rats were placed in a novel open field arena for locomotion assessment during both the light and dark periods. Inflammation and stress mediators were evaluated in plasma 2, 3, 5 or 14 h into the dark phase. Pre-treatment with SB-239063 significantly reversed the locomotor deficits induced by LPS injection. Interleukin (IL)-1β, IL-6, IL-10, Granulocyte-Macrophage-Colony Stimulating Factor, Interferon-γ, and C-reactive-protein levels were increased significantly by LPS, but not when LPS was preceded by SB-239063 treatment. LPS significantly decreased growth-hormone and Prolactin, and this effect was attenuated by SB-239063. Tumor Necrosis Factor-α, Adrenocorticotropic Hormone and Corticosterone levels were significantly higher in LPS-treated rats and were not normalized by SB-239063. Thus, we demonstrate that acute treatment with SB-239063 may have ameliorating effects in early changes of LPS-induced sickness behavior and alteration in the peripheral cytokines/hormones. As such, our procedure may offer an opportunity to test the activity of novel anti-inflammatory compounds on specific symptoms of sickness associated with neuroimmune dysfunctions.

1. Introduction

Peripheral injection of Lipopolysaccharide (LPS), a major structural component of the wall of Gram-negative bacteria, is a widely used experimental tool for the investigation of neuro-immune-endocrine responses and interactions. In preclinical models, a single exposure to LPS challenge may cause a marked increase in circulating cytokines, such as Interleukin (IL)-1β, IL-6, and Tumor Necrosis Factor (TNF)-α, as well as a characteristic set of physiological and behavioral responses (Anisman et al., 2005; Larson and Dunn, 2001; Yirmiya, 1996). Specifically, the syndrome elicited by LPS has been termed 'sickness behavior', since it comprises reductions in general activity, exploration, social interest, feeding, and body weight (Kelley et al., 2003; Shen et al., 1999). In addition to these effects, LPS functions as a stressor, by increasing the release of corticotrophin releasing factor (CRF) and adrenocorticotropic hormone (ACTH) from the hypothalamus–pituitary–adrenal (HPA) axis (Turnbull and Rivier, 1999), targeting pituitary hormone and adrenal corticosterone secretions (De Laurentiis et al., 2002; Priego et al., 2003; Suzuki et al., 1986; Vakharia and Hinson, 2005). Systemic administration of LPS to healthy human subjects causes effects comparable to those established in animals, including physical symptoms of sickness and increased secretion of pro-inflammatory cytokines (particularly IL-1, IL-6, TNF-α) as well as cortisol (Bahador and Cross, 2007; Lowry, 2005; Richardson et al., 1989).

A key role in mediating the inflammatory response in many cell types, including immune, inflammatory and endothelial cells, is played by the p38 mitogen-activated protein kinase (MAPK) pathway (Kumar et al., 2003; Schieven, 2009). p38 MAPK is activated in response to a variety of stimuli such as growth factors, environmental stressors, and inflammatory triggers, such as LPS or cytokines, like TNF-α or IL-1β (Lee and Young, 1996). The p38 MAPK is a 38kD polypeptide which belongs to a family of 4 isoforms (α, β, γ, δ) (Lee et al., 1994) whose first member was also identified on the basis of the rapid phosphorylation of the tyrosine residue in response to LPS stimulation (Han et al., 1994). Upon its activation p38 MAPK contributes either directly or indirectly to enhance the inflammatory response to cellular stress (Schieven, 2009). Therefore, p38 MAPK inhibitors have been proposed as therapeutic agents for a large number of diseases, all having an inflammatory and/or

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neurodegenerative component. The list includes rheumatoid arthritis, chronic obstructive pulmonary disease, severe asthma, psoriasis, Alzheimer's disease, and clinical depression (Mayer and Callahan, 2006; Miller and Raison, 2006; Munoz and Ammit, 2010). SB-239063, a second-generation p38 MAPK inhibitor, has shown efficacy in experimental models of stroke as well as inflammation (Barone et al., 2001; Legos et al., 2001, 2002; Underwood et al., 2000a,b). In particular, SB-239063 potently inhibits p38 MAPK catalytic activity (IC_{50} = 44 nM), corresponding to a strong inhibition of inflammatory responses in in vitro as well as in vivo systems (Underwood et al., 2000a,b). Thus, the aim of the present investigation was a time-course evaluation of SB-239063 in a LPS-induced sickness model, assessing the compound efficacy, following its acute administration on both psychomotor deficits and peripheral biomarkers related to immune and stress responses.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats (Charles River Labs, Calco, Italy), weighing between 270 and 310 g at the start of testing, were used in the present study. Animals were single-housed in standard propylene cages (Techniplast, Varese, Italy), lined with wood chip bedding, and were provided with ad libitum access to food pellets (Riepper, Bolzano, Italy) and tap water. Animals were acclimatized for at least 7 days in a room controlled for temperature (22 ± 1 °C), humidity (60%), and lighting (12 h light–dark cycle; lights on from 0600 h to 1800 h). On the day of the test all animals submitted to the testing procedures were transferred from housing to the experimental room to acclimatize for 1 h before drug administration.

All experiments were performed in accordance with European Community ethical regulations on the care of animals for scientific research (CEE Council 86/609 Italian D.L. 27/01/92 no. 116) and were fully compliant with GlaxoSmithKline ethical standards.

2.2. Treatments

LPS (derived from Escherichia coli 0111:B4, L-2630, lot. 034 K4105, Sigma-Aldrich, Milan, Italy) was dissolved in 0.9% isotonic saline (Fresenius Kabi, Verona, Italy) and sonicated for 5 min until complete solubilization. LPS was injected intraperitoneally (i.p., 2 ml/kg body weight) at 125 μg/kg. This dose of LPS was chosen since it causes marked signs of sickness behavior in the rat (Bison et al., 2009). SB-239063 [trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl) imidazole] was synthesized by the Department of Medicinal Chemistry at GlaxoSmithKline (UK) and was determined to be at least 99% pure as free base. The compound was suspended in a vehicle made of 0.5% Methylcellulose (w/v) (M0430 Sigma-Aldrich, Milan, Italy) containing 0.1% Tween 80 (v/v) (P1754, Sigma-Aldrich, Milan, Italy). SB-239063 was administered orally (p.o., 5 ml/kg body weight). Control rats were injected with the appropriate vehicle solutions.

2.3. Behavioral experiments

2.3.1. Acute effects of SB-239063 on locomotor activity

A first set of rats was randomly treated with either vehicle (n = 7) or SB-239063 at 3, 10 or 30 mg/kg (n = 8/group), and motor activity assessed 1 h following administration of the drug. The time of treatment was chosen according to the time to peak concentration of this drug in plasma (Ward et al., 2001). All treatments and motor activity assessment occurred into the light period phase (0800–1200 h).

2.3.2. Acute effects of SB-239063 on LPS-induced hypolocomotion

A second set of animals was randomly assigned to one of the following five groups of treatment (n = 7/group): the control group (Veh/Veh) received a pre-treatment with 0.5% Methocel/0.1% Tween80, followed by saline; the LPS group (Veh/LPS) received a pre-treatment with 0.5% Methocel/0.1% Tween80, followed by LPS administration; the SB-239063 groups (SB 3/LPS, SB 10/LPS and SB 30/LPS) received a pre-treatment of 3, 10 or 30 mg/kg SB-239063, followed by LPS. Two hours following either LPS or saline injections, animals were assessed for their locomotor activity. All treatments and motor activity assessment occurred in the light phase (0800–1300 h).

2.3.3. Long-term effects of SB-239063 on LPS-induced hypolocomotion

A third set of animals was randomly distributed to one of the following four groups of treatment (n = 8–9/group): 0.5% Methocel/0.1% Tween80, followed by saline 0.5% Methocel/0.1% Tween80, followed by LPS administration; either 10 or 30 mg/kg SB-239063 pre-treatment, followed by LPS. Two hours after LPS or saline injections, animals were assessed for locomotor activity during the dark phase (1800–0600 h).

2.3.4. Animal locomotor activity measurements

Locomotor activity was measured in non-habituated rats using 42 cm × 42 cm × 30 cm VersaMax Animal Activity Monitors (Model NVMA16TT/W, Accuscan Instruments Inc., Columbus, USA). Animal activity was recorded as total distance (cm). For the first and second set of animals the total distance was recorded in 5-min bins during 30 min; for the third set of animals the total distance was recorded in 30 min time blocks during 720 min (12 h).

2.4. Biomarker time-course assessment

An additional set of animals was used to assess the temporal effects of SB-239063 on LPS-induced alteration in peripheral analytes. In order to reduce the number of animals the time-course study was performed by repeated blood collections. On the day of the test, rats were split in groups and received a treatment as described in Section 2.3.3.

2.4.1. Surgery

Rats (n = 8/group) were anesthetized under isofluorane (Abbott, Milan, Italy) 2.5% in 100% O_2, then administered subcutaneously with either Rubrocillina Veterinaria (Intervet Productions, Aprilia, Italy) at 0.1 ml/rat (25.000 U/l) or Rimadyl (Pfizer, Milan, Italy) at 5 mg/ml (1 ml/kg body weight). A peripheral vascular catheter (PVC, 0.5 mm ID 0.9 mm OD, Instech Solomon, Plymouth Meeting, USA) was inserted in the left femoral vein, tunneled to the interscapular region where it was exteriorized; then filled with a lock solution (Glucose 50%, Heparine 500 UI/ml in saline) (Sigma-Aldrich, Milan-Italy and Sirton Pharmaceuticals, Como-Italy, respectively). To protect the catheter rats were dressed with a Covance harness (Instech Solomon, Plymouth Meeting, USA). Rats were allowed to recover from surgery for at least 5–7 days. During this period catheters were regularly checked for patency and the body weight of animals recorded.

2.4.2. Blood sampling

Two hours before the study starts, rats were connected through a tether to an automated sampling device (AccuSampler® Automated Blood Sampling syste; DiLab, Lund, Sweden) which allows animal freedom of movement. The first blood collection was performed at 1440 h (base-line time-point), rats were then administered SB-239063 or vehicle (1500 h). At 1540 h a pre-LPS blood collection was carried out (pre-challenge time-point), followed by LPS or saline treatment at 1600 h. Next blood collections were performed at 1800 h (2 h after LPS or saline treatment); at 1900 h (3 h), at 2100 h (5 h) and at 0600 h (14 h). At each time-point, 300 μL blood samples (2 × 150 μL, 7 min gaps) were collected into tubes containing 10 μL 200 mM K_3EDTA (Sigma-Aldrich, Milan, Italy), 3 μL Protease Inhibitor Cocktail (Sigma-Aldrich, Milan, Italy), 3 μL DPPIV Inhibitor (Millipore, Billerica, USA) and centrifuged 10 min at 1800 g at 4 °C. Supernatant was split into aliquots

and stored at −80 °C; an aliquot was added with indomethacin (Sigma-Aldrich, Milan, Italy) to a final concentration of 10 μg/ml.

2.4.3. Quantitative determination of plasma analyte levels

Corticosterone levels were measured with ImmunoChem™ [125I] corticosterone RadioImmunoAssay (MP Biomedicals, LLC, Diagnostic Division, Irvine, USA). Prostaglandin E2 (PGE2) was measured in the indomethacin-containing aliquot with an ELISA kit (R & D System, USA), following manufacturer's instructions. The other analytes were measured by Luminex technology on a BioPlex instrument (Bio-Rad, Hercules, USA) with Bio-Rad or Milliplex kits (Millipore) following manufacturer's instructions. CRP was measured with the Mouse/Rat CRP-1 Plex kit (Millipore); ACTH, GH, prolactin, and Brain-derived neurotrophic factor (BDNF) were analyzed with the Rat Pituitary Panel (Millipore); IL1-β, IL-6, IL-10, TNF-α, IFN-γ, GM-CSF, were measured with the Rat Cytokine kit (Bio-Rad).

2.5. Statistical analysis

Statistical analyses were conducted using Statistica v8 (Stat Soft Inc., 1984–2007, Tulsa, USA). Locomotor activity data were analyzed using a One-way Analysis of Variance (ANOVA) followed by LSD *post hoc* tests. For animals tested during the dark phase, the data were split into 4 time intervals (0–30 min, 31–90 min, 91–270 min and 271–720 min) and analyzed by one-way ANOVA followed by LSD post hoc test. This allowed the first period of animal exploration phase (0–30 min) to be distinguished from the subsequent ones (31–90 min, 91–270 min and 271–720 min) and to simplify the comparison of locomotor activity data with data originating from the biomarker experiment. A comprehensive comparison between locomotor activity data and biomarker data was not possible due to the temporal differences between the data collection methods (i.e., locomotor activity measures reflect behavior occurring over a fixed time interval, whilst biomarker data reflect a precise time point at which the blood was sampled). Thus, we have analyzed locomotion data according to 4 time intervals: 0–30 min (2 and 2.5 h after LPS or saline injections), matching the 2 h sampling time in the biomarker experiment; 31–90 min, (2.5–3.5 h after LPS or saline injection), within includes the 3 h blood sampling time; 91–270 min (between 3.5 and 6.5 h after LPS or saline injection) including the 5 h sampling time for biomarkers; 271–720 min (between 6.5 and 14 h following LPS or saline injection) corresponding to 14 h blood collection timing. Two-way ANOVAs with time and treatment as main factors and plate as additional factor were carried out for biomarker measurements and were followed by planned comparisons. Data were log transformed when needed to satisfy normality assumptions of ANOVA. Results from all studies were expressed as the observed mean ± standard error of the mean (S.E.M.). In all studies, levels of statistical significance were set at $P<0.05$, while results with $P<0.10$ were considered marginally significant.

3. Results

3.1. Acute effects of SB-239063 on locomotor activity

No statistically significant effect of treatment on total distance was observed [$F_{(3,27)}=0.63$, $P=0.6$] (Fig. 1).

3.2. Acute effects of SB-239063 on LPS-induced hypolocomotion

No significant effect of treatment was observed [$F_{(4,29)}=1.87$, $P=0.142$; Fig. 2]. However, post hoc comparisons revealed that the reduction of motor activity in animals treated with LPS only was close to significance level when compared to the control group ($P=0.053$), as Veh/LPS rats traveled approximately 30% of Veh/Veh rats (Fig. 2). SB 10/LPS and SB 30/LPS rats traveled significantly longer distances than Veh/LPS animals ($P<0.05$ each; Fig. 2) and did not differ with respect to Veh/Veh rats.

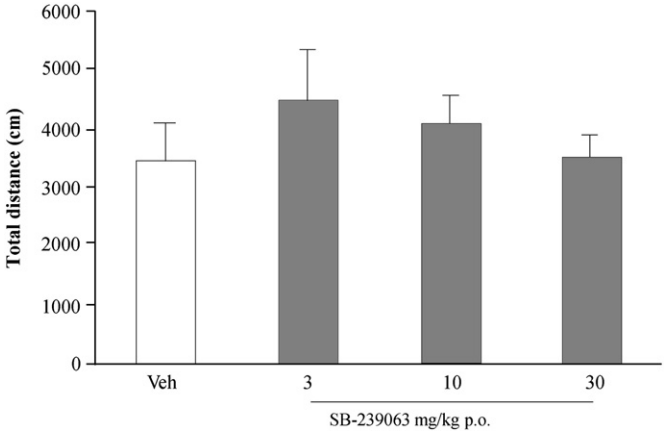

Fig. 1. Acute effect of SB-239063 on locomotor activity. Group means of total distance (cm) values traveled by rats during the light phase period (0800–1200 h) following treatments with either vehicle (n = 7) or SB-239063 at 3, 10 or 30 mg/kg (n = 8/group). One hour following drug administration, rats were placed in the automated activity cages with ambulation assessed for a total of 30 min.

3.3. Long-term effects of SB-239063 on LPS-induced hypolocomotion

A statistically significant effect of treatment was observed at each time intervals considered [$F_{(3,31)}=9.45$, $P<0.001$ for 0–30 min; $F_{(3,31)}=9.15$, $P<0.001$ for 31–90 min; $F_{(3,31)}=6.53$, $P=0.001$ for 91–270 min and $F_{(3,31)}=10.99$, $P<0.001$ for 271–720; Fig. 3]. In particular, the Veh/LPS group showed a significantly lower activity than Veh/Veh across the whole 12 h dark period examined ($P<0.001$; Fig. 3). The animals in the SB 10/LPS group did not differ from Veh/LPS animals at any time interval examined (Fig. 3), while they showed significantly lower values compared to the Veh/Veh group ($P<0.01$; Fig. 3). On the contrary, animals in the SB 30/LPS group showed significantly higher locomotion scores during the first 30 min compared to Veh/LPS ($P<0.01$; Fig. 3). In addition, the motor activity of SB 30/LPS treated animals compared to Veh/LPS remained significantly higher also throughout the late test phases ($P<0.05$; Fig. 3). However, their level of activity was found to be significantly different from Veh/Veh rats ($P<0.05$).

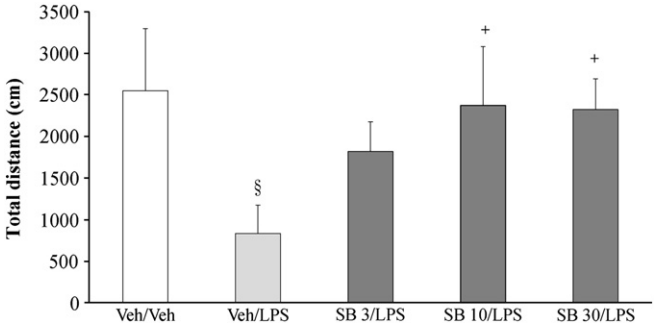

Fig. 2. Acute effect of SB-239063 on LPS-induced hypolocomotion. Group means of total distance (cm) values moved by rats whose activity was monitored during the light phase period (1000–1300 h) following treatment with either vehicle (Veh/Veh) or LPS (Veh/LPS) or SB-239063 at 3, 10 or 30 mg/kg plus LPS (SB 3/LPS; SB 10/LPS and SB 30/LPS) (n = 7/group). SB-239063 was administered 1 h prior LPS challenge. Rats were placed in the automated activity cages 2 h post LPS challenge with locomotion monitored for a total period of 30 min. Significant differences determined by post hoc LSD test: §$P=0.05$ in the comparison Veh/Veh vs. Veh/LPS; +$P<0.05$ in the comparison Veh/LPS vs. SB (10 or 30)/LPS. Error bars represent S.E.M.

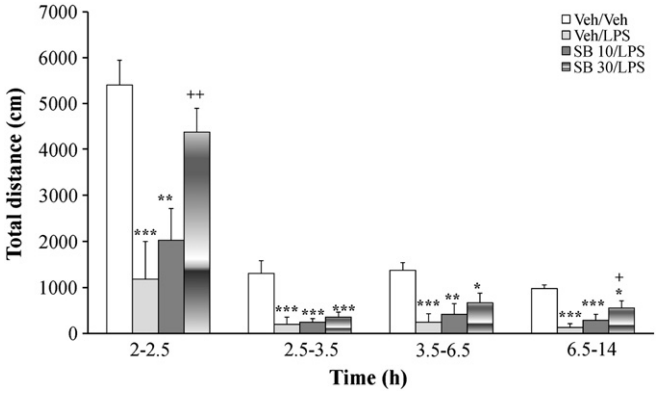

Fig. 3. Long-term effects of SB-239063 on LPS-induced hypolocomotion. Group means of total distance (cm) values moved by rats tested during the dark period (light off 1800 h–light on 0600 h) following treatment with either vehicle (Veh/Veh) or LPS (Veh/LPS) or SB-239063 at 10 or 30 mg/kg plus LPS (SB 10/LPS and SB 30/LPS). SB-239063 was administered 1 h prior LPS challenge. Rats were tested immediately before the dark phase onset (2 h post LPS challenge) with locomotion monitored for 12 h (30 min blocks for a total of 720 min). Locomotor data were split into 4 intervals: 0–30 min, 31–90 min, 91–270 min, 271–720 min and corresponding to 2–2.5 h, 2.5–3.5 h, 3.5–6.5 h and 6.5–14 h following LPS or saline injection. Significant differences determined by post hoc LSD test: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. Veh/Veh; $++P<0.01$ in the comparison Veh/LPS vs. SB (10 or 30)/LPS; $+P<0.05$ in the comparison Veh/LPS vs. SB (10 or 30)/LPS. Error bars represent S.E.M.

3.4. Inflammation analytes

Treatment with SB-239063 alone did not significantly alter the levels of any inflammation marker (baseline values versus pre-challenge values; data not shown).

In contrast, after LPS administration, the levels of inflammation analytes varied with treatment (at least for some time-points), showing statistically significant effects of treatment or significant treatment × time interactions.

Plasma IL-1β levels were affected by treatment [treatment: $F_{(3,28)}=2.71$, $P=0.06$; interaction: $F_{(9,84)}=4.61$, $P<0.001$]; post-hoc tests revealed a significant increase in the Veh/LPS group compared to the Veh/Veh group after 2 h ($P<0.01$), 3 h ($P<0.01$) and 5 h ($P<0.05$), returning similar to control levels after 14 h (Fig. 4A). The increase induced by LPS was reversed significantly by 30 mg/kg SB-239063 at the 2 h time-point ($P<0.05$ vs. Veh/LPS; Fig. 4A). LPS treatment alone induced a large increase in IL-6 levels [treatment: $F_{(3,28)}=5.63$, $P=0.004$; interaction: $F_{(9,84)}=3.18$, $P=0.002$], which was statistically significant at all time-points measured ($P<0.01$ at 2 h, $P<0.001$ at 3 h; $P<0.01$ at 5 h; $P<0.05$ at 14 h in post-hoc comparisons vs. Veh/Veh; Fig. 4B). Although the increase peaked 2 h after LPS treatment, both 10 and 30 mg/kg SB-239063 were able to reverse significantly the increase at this time-point ($P<0.05$ vs. Veh/LPS; Fig. 4B). IL-10 levels were elevated significantly by LPS treatment alone [treatment: $F_{(3,26)}=3.67$, $P=0.02$, interaction: $F_{(9,83)}=1.97$, $P=0.053$] at 2 h ($P<0.001$), 3 h ($P<0.05$) and 5 h ($P<0.001$) (Fig. 4C). The increase was reversed significantly by 10 mg/kg SB-239063 at the 2 h and 5 h time-points ($P<0.05$ vs. Veh/LPS; Fig. 4C) and a trend towards reduction was detected at the 3 h time-point ($P=0.07$; Fig. 4C). Treatment with 30 mg/kg SB-239063 reversed significantly the increase in IL-10 2 h after treatment ($P<0.01$ vs. Veh/LPS; Fig. 4C). For IFN-γ, a statistically significant interaction effect was observed [treatment: $F_{(3,28)}=2.00$, $P=0.13$; interaction: $F_{(9,84)}=3.03$, $P=0.003$]: in particular, LPS alone significantly augmented IFN-γ levels 2 h after treatment ($P<0.05$ compared to Veh/Veh group) and a similar trend could be detected at the 3 h and 5 h time-points ($P=0.060$ and $P=0.055$, respectively; Fig. 4D). This rise was blocked by 30 mg/kg SB-239063 ($P<0.01$ vs. Veh/LPS; Fig. 4D) at the 2 h time-point. TNF-α levels were elevated significantly in Veh/LPS group compared to Veh/Veh [treatment: $F_{(3,28)}=4.75$, $P=0.008$; interaction: $F_{(9,84)}=3.63$, $P<0.001$] at the early time-points ($P<0.01$ at 2 h; $P<0.05$ at 3 h; $P<0.05$ at 5 h; Fig. 5A) and returned to control levels after 14 h. No statistically significant differences were observed due to SB-239063 treatment (Fig. 5A). GM-CSF levels increased after LPS treatment alone [treatment: $F_{(3,28)}=5.79$, $P=0.003$; interaction: $F_{(9,84)}=1.82$, $P=0.08$], reaching statistical

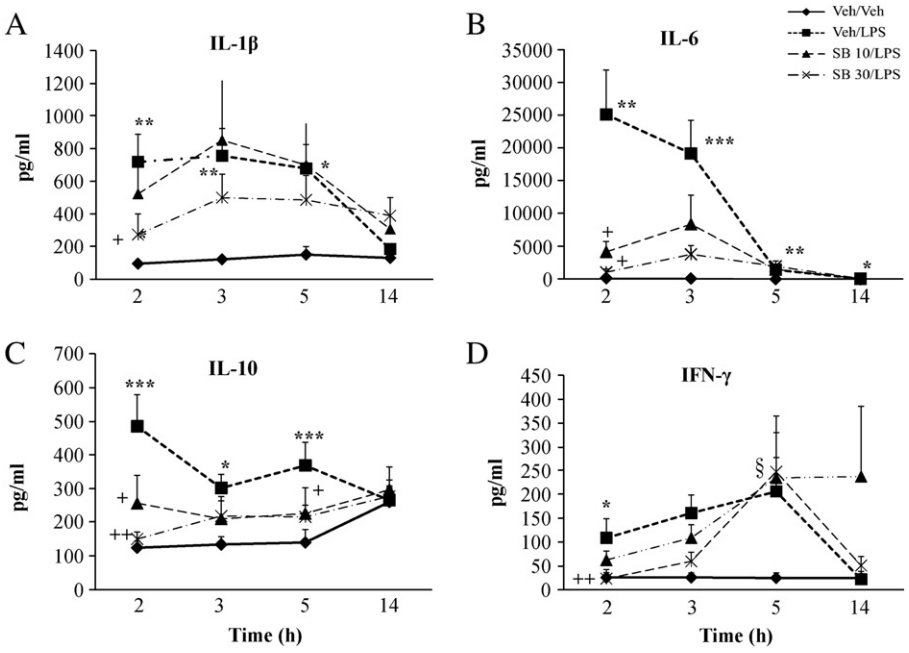

Fig. 4. Long-term effects of SB-239063 on LPS-stimulated cytokine levels. Mean concentration plasma levels IL-β (A), IL-6 (B), IL-10 (C), and IFN-γ (D) of rats treated with either vehicle (Veh/Veh) or LPS (Veh/LPS) or SB-239063 at 10 and 30 mg/kg plus LPS (SB 10/LPS and SB 30/LPS) 2, 3, 5 and 14 h post LPS injection (n = 8/group) during the dark period (i.e., dark onset–1800 h). SB-239063 was administered 1 h prior LPS challenge. Significant differences determined by post hoc analysis: $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ in the comparison vs. Veh/Veh; §$P=0.05$ in the comparison Veh/Veh vs. Veh/LPS; $++P<0.01$ in the comparison Veh/LPS vs. SB (10 or 30)/LPS; $+P<0.05$ in the comparison Veh/LPS vs. SB (10 or 30)/LPS. Error bars represent S.E.M.

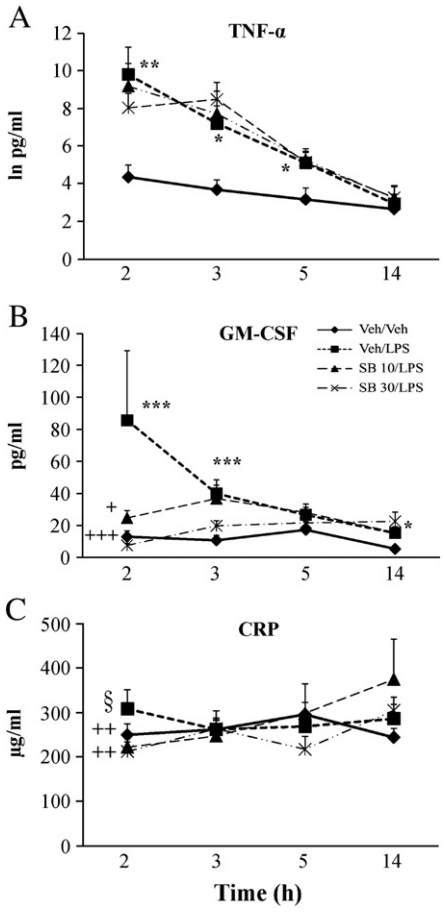

Fig. 5. Long-term effects of SB-239063 on LPS-stimulated inflammatory analyte levels. Mean concentration plasma levels of TNF-α (A), GM-CSF (B), and CRP (C) of rats treated with either vehicle (Veh/Veh) or LPS (Veh/LPS) or SB-239063 at 10 and 30 mg/kg plus LPS (SB 10/LPS and SB 30/LPS) 2, 3, 5 and 14 h post LPS injection (n = 8/group) during the dark period (i.e., dark onset–1800 h). Significant differences determined by post hoc analysis: ***$P<0.001$, **$P<0.01$, *$P<0.05$ in the comparison Veh/Veh vs. Veh/LPS; §$P=0.05$ in the comparison Veh/Veh vs. Veh/LPS; +++$P<0.001$, ++$P<0.01$, +$P<0.05$ in the comparison Veh/LPS vs. SB (10 or 30)/LPS. Error bars represent S.E.M.

significance in post-hoc comparisons vs. Veh/Veh at 2 h ($P<0.001$), 3 h ($P<0.001$) and 14 h ($P<0.05$; Fig. 5B). A reversal was observed after 2 h with both doses of SB-239063 ($P<0.05$ vs. Veh/LPS at 10 mg/kg, $P<0.001$ vs. Veh/LPS at 30 mg/kg; Fig. 5B). For CRP, a statistically significant interaction was identified [treatment: $F_{(3,26)}=0.68$, $P=0.57$; interaction: $F_{(9,78)}=2.52$, $P=0.01$]. In particular, LPS treatment per se increased CRP levels at 2 h after administration ($P=0.052$ vs. Veh/Veh in post hoc test; Fig. 5C). At the same time-point, both doses of SB-239063 induced a statistically significant reduction of CRP levels compared to Veh/LPS ($P<0.01$; Fig. 5C). A time effect was also detected [$F_{(3,78)}=3.80$, $P=0.01$], with higher levels in the morning (14 h) compared to the evening time-points ($P<0.05$). No variation in PGE2 plasma levels was induced by LPS or p38 inhibitor treatment (data not shown); a time effect was detected [$F_{(3,78)}=5.50$, $P=0.002$], with low levels at 3 h and high values at 14 h ($P<0.001$). IL-2 levels were not significantly altered by LPS administration or by treatment with SB-239063 (data not shown); a time effect was detected [$F_{(3,78)}=12.22$, $P<0.001$], showing that IL-2 levels at 14 h were significantly lower than at the other time-points measured.

3.5. Hormones

Treatment with SB-239063 alone did not modify plasma levels of any hormones measured (data not shown). In the ANOVA, ACTH levels showed the expected time effect [$F_{(3,84)}=35.06$, $P<0.001$], with significantly lower levels in the morning (14 h) in comparison with evening values as revealed by post hoc test (2 h, 3 h, $P<0.001$). In addition, ACTH levels varied with treatment [$F_{(3,28)}=3.57$, $P=0.03$]: post hoc analysis revealed that at the time-points 2 h ($P<0.01$), 3 h ($P<0.05$) and 5 h ($P<0.05$, Fig. 6A) ACTH concentration was significantly higher for Veh/LPS than Veh/Veh and was not affected by SB-239063. A similar pattern was observed for plasma corticosterone: a time effect [$F_{(3,84)}=27.79$, $P<0.001$] related to the circadian decrease in the morning (14 h) in comparison with evening values (2 h, $P<0.001$) was revealed. In addition, a treatment effect was detected [$F_{(3,28)}=5.10$, $P=0.006$], due to LPS-induced augment at 2 h ($P<0.001$), 3 h ($P<0.001$), 5 h ($P=0.054$) and 14 h ($P<0.05$; Fig. 6B) as shown in post hoc comparisons. No statistically significant modification was induced by SB-239063 treatment ($P>0.05$ vs. Veh/LPS; Fig. 6B). A statistically significant effect of treatment or treatment × time interaction was found for GH levels [treatment: $F_{(3,26)}=3.15$, $P=0.04$; interaction: $F_{(9,78)}=2.20$, $P=0.03$]: post-hoc tests revealed a significant decrease in the Veh/LPS compared to the Veh/Veh at 2 h and 3 h after administration ($P<0.001$ and $P<0.01$, respectively; Fig. 6C). Treatment with both doses of SB-239063 significantly diminished the reduction at the 2 h time-point ($P<0.05$ vs. Veh/LPS at 10 mg/kg and $P<0.05$ vs. Veh/LPS at 30 mg/kg; Fig. 6C). A similar effect was found for prolactin levels [treatment $F_{(3,26)}=3.45$, $P=0.03$; interaction: $F_{(9,82)}=2.63$, $P=0.01$] with significantly lower levels in the Veh/LPS than those shown by Veh/Veh at the 3 h and 5 h time-points ($P<0.05$; Fig. 6D). A trend towards decreased prolactin levels could also be appreciated at the 2 h time-point, although it was not statistically significant. At this time-point, rats treated with SB 10/LPS showed significantly higher prolactin levels as compared to the Veh/LPS ($P<0.05$; Fig. 6D), thus suggesting that the trend towards LPS-induced reduction could be reversed by p38 inhibition. No difference in BDNF levels was associated to treatment groups. An effect of time was revealed [$F_{(3,84)}=11.98$, $P<0.001$], with significantly lower levels detected at the 14 h time-point in comparison with the evening samplings (data not shown).

4. Discussion

The present results demonstrate that efficacy of an acute treatment with the p38 MAPK inhibitor SB-239063 in a rat model of LPS-induced sickness behavior. SB-239063 was tested at 10 or 30 mg/kg, both doses chosen on the basis of previous preclinical studies showing their efficacy in inflammatory models to produce full inhibition of p38 MAPK activity (Underwood et al., 2000a,b).

The current experiments revealed that SB-239063 efficacy depended upon testing conditions (light vs. dark phase). During the light period, both doses of SB-239063 prevented the motor reduction induced by LPS up to 2.5 h following the endotoxin challenge, although no dose-dependent effects of SB-239063 were detectable. Importantly, SB-239063 alone did not alter the general motor activity in rats. During the dark phase, only the higher dose (30 mg/kg) of SB-239063 corresponded to behavioral protection against the motor impairment due to LPS; furthermore this beneficial effect was observed maximally 2.5 h after LPS but it was not sustained at later time points. This short-lived effect is in agreement with the pharmacokinetic profile of this compound reported in previous studies (Ward et al., 2001), showing that oral administration leads to plasma concentrations of SB-239063 peaking within 1 h after dosing and then rapidly declining. Thus, it can be assumed that the excellent in vivo protection profile of SB-239063 on locomotor deficits could be observed only in correspondence of the maximal bioavailability levels of SB-239063 in plasma, while disappearing at later time points due to the fairly rapid metabolism of the compound. It is worth emphasizing that SB-239063 showed greater efficacy when testing occurred during the light procedure, as the lower dose was also found to be active. It is likely that circadian variations in pharmacokinetic parameters could explain the observed effect. Previous studies (Sajan et al., 2009) showed how the pharmacokinetics of many

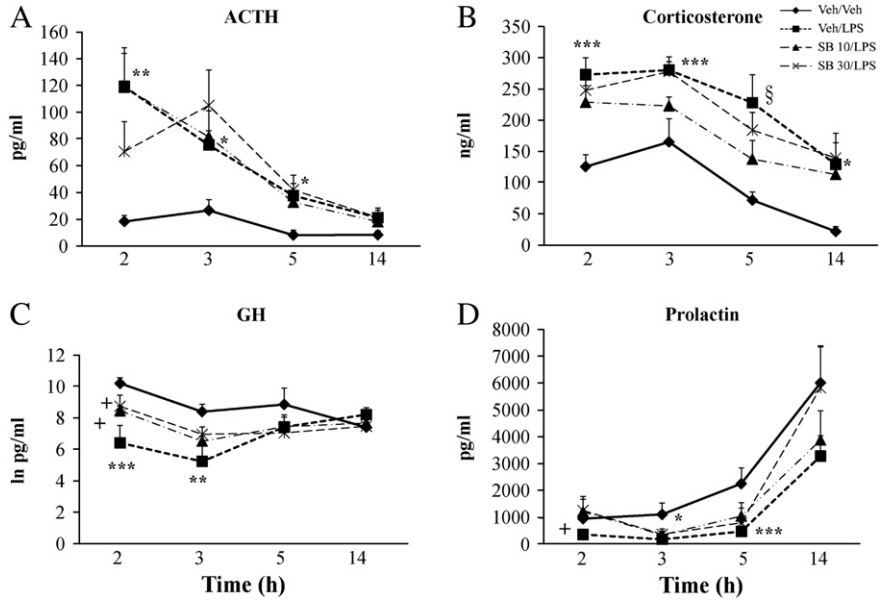

Fig. 6. Long-term effects of SB-239063 on LPS-stimulated hormone levels. Mean concentration plasma levels of ACTH (A), Corticosterone (B), GH (C), and Prolactin (D) of rats treated with either vehicle (Veh/Veh) or LPS (Veh/LPS) or SB-239063 at 10 and 30 mg/kg plus LPS (SB 10/LPS and SB 30/LPS) 2, 3, 5 and 14 h post LPS injection (n = 8/group) during the dark period (i.e., dark onset–1800 h). Significant differences determined by post hoc analysis: ***P<0.001, **P<0.01, *P<0.05 in the comparison Veh/Veh vs. Veh/LPS; §P = 0.05 in the comparison Veh/Veh vs. Veh/LPS; +P<0.05 in the comparison Veh/LPS vs. SB (10 or 30)/LPS. Error bars represent S.E.M.

commercial drugs may be influenced by physiological functions displaying circadian rhythms (i.e., changes in gastric motility), thus explaining differences in the bioavailability of compound across the day/night cycle.

The observed behavioral response to the LPS challenge was also paralleled by the increase in circulating levels of inflammatory mediators, such as IL-1β, IL-6, TNF-α, IL-10, IFN-γ, GM-CSF, CRP, as well as stress hormones (ACTH and corticosterone), peaking rapidly following LPS challenge. Interestingly, SB-239063 suppressed the LPS-induced elevation in plasma levels of multiple inflammatory markers, including IL-1β, IL-6, IL-10, IFN-γ, GM-CSF and CRP; this effect was maximal 2 h after LPS challenge and it slowly declined at later time-points, corresponding to the temporal pattern of the behavioral effects as well as to the pharmacokinetic profile of the compound. On the other hand, SB-239063 had no effect on the release of TNF-α and stress hormones elicited by LPS. Several lines of evidence have documented the anti-inflammatory action of SB-239063, both in vitro and in vivo, many of which based on the basis of the inhibition of LPS-induced TNF-α production (Barone et al., 2001; Underwood et al., 2000a,b). However, despite sharing some commonalities for the cytokine-suppressive effect of SB-239063 reported by these authors, in the present study SB-239063 had no efficacy on the LPS-induced augmentation of circulating TNF-α. Similarly, SB-239063 did not normalize the rise in ACTH and corticosterone induced by LPS. Whereas procedural differences (i.e., different fragment of LPS and rat strain, differences in drug treatment and biomarker assessment technique) may account for the observed discrepancy, further data are needed to explain these results. However, the simultaneous lack of efficacy of SB-239063 towards the LPS-induced changes in TNF-α, ACTH, and corticosterone, should be considered in light of the influence of pro-inflammatory cytokines on glucocorticoids during inflammation (Besedovsky and Del Rey, 2000; Tsigos and Chrousos, 2002). In particular, in vitro studies showed that both TNF-α and IL-1β activate the hypothalamo-pituitary-adrenal axis with an enhanced secretion of adrenocorticotropic hormone and corticosterone for modulating the inflammatory response (Escher et al., 1997). Additionally, a crucial role for TNF-α in triggering the early, peak phase of ACTH secretion after a challenge of LPS has been demonstrated in several in vivo studies (Kakizaki et al., 1999; Perlstein et al., 1993; Takao et al., 1997).

Greater attention has been paid to the role played by p38 MAPK inhibition on the LPS-induced increases in IL-10 levels. In agreement with the present results, similar findings have been described despite the use of different p38 MAPK inhibitors in in vitro systems, showing that the pharmacological inhibition of p38 MAPK may result in the abrogation of LPS-induced IL-10 production (Foey et al., 1998). It is commonly accepted that IL-10 acts as an anti-inflammatory cytokine due to its ability to suppress the synthesis of several pro-inflammatory cytokines including IL-1, IL-6, TNF-α (Fiorentino et al., 1991). Despite the functional role of altered IL-10 levels on behavior has still to be fully elucidated, our results showed that the behavioral protection offered by SB-239063 did occur although the levels of IL-10 were within control levels. These results suggest that the behavioral effects of SB-239063 are not the consequence of IL-10 anti-inflammatory properties and are likely associated with a more general anti-inflammatory effect of the compound itself.

In this study, reduced GH and prolactin levels were also observed after LPS treatment. Both prolactin and GH are members of hemopoietin/cytokine family and are involved in immune regulation (Bazan, 1989; Redelman et al., 2008), although the mechanisms by which GH and prolactin mediate this response is still far from being comprehensively understood (Clevenger et al., 1998; Gent et al., 2003). Nevertheless, in rats, acute LPS treatment has been repeatedly associated to a reduction in GH blood levels (Kasting and Martin, 1982; Soto et al., 1998), in agreement with the present findings. Likewise, consistently with present findings, reductions in blood prolactin levels have been demonstrated after exposure to bacterial endotoxin in rats and mice (Bik et al., 2004; De Laurentiis et al., 2002; Hollis et al., 2005). LPS affects pituitary hormone secretion directly and indirectly by inducing cytokine release. The LPS-induced increase in TNF-α secretion is responsible for an increase dopamine turnover in the HPA axis, which, in turn could mediate the inhibitory effect of LPS and TNF-α on prolactin release (De Laurentiis et al., 2002). Data show that the effects of LPS on GH secretion are consistent with peripheral cytokine presentation to the pituitary, as well as with a potential direct action on selective populations of pituitary cells via CD14, the LPS receptor (Daniel et al., 2005). SB-239063 ameliorated the LPS-induced alterations on both pituitary hormones; nevertheless, it would

be highly speculative and beyond the scope of the present experiments to attempt to define the molecular mechanisms involved in the observed modulation. Further studies are warranted to elucidate the complex pathways linking general immune response to pituitary hormone release and p38 MAPK inhibition.

Considering the overall time-course of the reported alterations in behavior and peripheral biomarkers, our results provide the first evidence that inhibition of the p38 MAPK pathway may have protective effects in the early components of 'sickness behavior', namely psychomotor retardation, and that these responses could be normalized notwithstanding high levels of TNF-α and stress hormones, leading to speculate that other inflammatory and stress mediators might be implicated. Consistent with our findings, it has been shown that pro-inflammatory cytokines, particularly IL-1 and IL-6, play a major role in regulating central nervous system-mediated suppression of physical activity in animal studies (Harden et al., 2006, 2008; Larson and Dunn, 2001; Netea et al., 2007). Psychomotor slowing and fatigue have also been reported to be elicited in human subjects in general inflammatory conditions, such as the one experimentally elicited by administration of endotoxin (Bahador and Cross, 2007).

Overall, the present study should not be considered as a simple additional evidence to the growing number of demonstrations of the anti-inflammatory activity of p38 MAPK inhibitors, but it could be seen in the light of broader implications. First, the present approach could be utilized for direct translation of pre-clinical results into early clinical assessment (i.e., dosage regimen in human subjects), given that LPS administration to normal subjects has become a common procedure to obtain proof of the principle for the action of anti-inflammatory compounds in clinical Phase I trials (Branger et al., 2002). Secondly, our procedure shows that specific symptoms associated with neuroimmune dysfunctions (i.e. fatigue, psychomotor disturbances) could be reversed by anti-inflammatory compounds. In conclusion, the present study demonstrates the efficacy of the p38 MAPK inhibitor SB-239063 on rat LPS-induced 'sickness behavior'; this activity is most likely to be mediated through the compound's anti-inflammatory properties that were assessed alongside the behavioral syndrome. These results therefore add to the growing body of evidence in support of pursuing p38 MAPK as a potential drug target and that selective pharmacological inactivation of this kinase may influence both central and peripheral pathways.

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