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Granulocyte-Colony Stimulating Factor (G-CSF) induces mechanical hyperalgesia via spinal activation of MAP kinases and PI_3K in mice

Thacyana T. Carvalho [a,1], Tamires Flauzino [a,1], Eliane S. Otaguiri [a,1], Ana P. Batistela [a], Ana C. Zarpelon [a], Thiago M. Cunha [b], Sérgio H. Ferreira [b], Fernando Q. Cunha [b], Waldiceu A. Verri Jr. [a,*]

[a] Departamento de Patologia, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid KM480 PR445, CEP 86051-990, Cx Postal 6001, Londrina, Paraná, Brazil

[b] Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Avenida Bandeirantes, 3900, CEP 14049-900, Ribeirao Preto, Sao Paulo, Brazil

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ABSTRACT

Granulocyte-colony stimulating factor (G-CSF) is a current pharmacological approach to increase peripheral neutrophil counts after anti-tumor therapies. Pain is most relevant side effect of G-CSF in healthy volunteers and cancer patients. Therefore, the mechanisms of G-CSF-induced hyperalgesia were investigated focusing on the role of spinal mitogen-activated protein (MAP) kinases ERK (extracellular signal-regulated kinase), JNK (Jun N-terminal Kinase) and p38, and PI_3K (phosphatidylinositol 3-kinase). G-CSF induced dose (30–300 ng/paw)-dependent mechanical hyperalgesia, which was inhibited by local post-treatment with morphine. This effect of morphine was reversed by naloxone (opioid receptor antagonist). Furthermore, G-CSF-induced hyperalgesia was inhibited in a dose-dependent manner by intrathecal pre-treatment with ERK (PD98059), JNK (SB600125), p38 (SB202190) or PI_3K (wortmanin) inhibitors. The co-treatment with MAP kinase and PI_3K inhibitors, at doses that were ineffective as single treatment, significantly inhibited G-CSF-induced hyperalgesia. Concluding, in addition to systemic opioids, peripheral opioids as well as spinal treatment with MAP kinases and PI_3K inhibitors also reduce G-CSF-induced pain.

1. Introduction

The colony-stimulating factors (CSFs) are a group of cytokines that support the survival, proliferation, differentiation, and end cell function of myeloid cells (Metcalf, 1993). For instance, granulocyte-CSF (G-CSF) enhances the production of neutrophils by inducing the proliferation and differentiation of its myeloid progenitor (Welte et al., 1985; Demetri and Griffin, 1991). G-CSF also activates terminally differentiated neutrophils by enhancing antibody-dependent killing, phagocytic activity and priming the respiratory burst (Bober et al., 1995). In agreement, G-CSF and G-CSF receptor deficient mice are severely neutropenic and susceptible to infections (Lieschke et al., 1994; Liu et al., 1996; Zhan et al., 1998; Battiwalla and McCarthy, 2009). Therefore, G-CSF plays an essential role in steady-state neutrophil production and in acute/rapid granulopoiesis during infections (Zhan et al., 1998).

The most important applicability of G-CSF in human therapy is related to granulopoiesis. The G-CSF treatment increases neutrophil counts in patients receiving myelosuppressive chemotherapy, patients with acute myeloid leukemia receiving induction or consolidation chemotherapy, patients receiving bone marrow transplant, patients undergoing peripheral blood progenitor cell collection and therapy, and patients with severe chronic neutropenia (Granulokine®, Filgrastim package insert; Dale et al., 1993; Battiwalla and McCarthy, 2009).

Nevertheless, the G-CSF treatment induces some side effects. For instance, according to a retrospective analysis of 341 healthy donors, the main adverse events of G-CSF therapy (Granulokine®, Filgrastim package insert) are pain (84%), headache (54%), fatigue (31%) and nausea (13%) (Battiwalla and McCarthy, 2009). In fact, G-CSF treatment induces bone, musculoskeletal and visceral pain in healthy volunteers and cancer patients (Granulokine®, Filgrastim package insert; Battiwalla and McCarthy, 2009). Thus, pain is the main side effect of G-CSF therapy.

The main therapy used to control G-CSF-induced pain is the treatment with opioids such as morphine (Granulokine®, Filgrastim

* Corresponding author. Present address: Departamento de Patologia, Universidade Estadual de Londrina, Rod. Celso Garcia Cid KM480 PR445, CEP 86051-990, Cx Postal 6001, Londrina, Paraná, Brazil. Tel.: +55 43 3371 4979; fax: +55 43 3371 4387.
E-mail addresses: waverri@uel.br, waldiceujr@yahoo.com.br (W.A. Verri).

[1] T.T.C., T.F. and E.S.O. contributed equally to this study.

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package insert). However, prolonged treatment might induce dependence because of opioid receptors desensitization leading to increasing doses of morphine. Moreover, the side effects of morphine include nausea, constipation, somnolence/sedation, and respiratory failure (Devulder et al., 2009). Evidence suggests spinal inhibition of pain processing as a successful strategy to reduce pain with reduced incidence of systemic side effect. The spinal inhibition of mytogen-activated protein (MAP) kinases p38, JNK (Jun N-terminal Kinase) and ERK (extracellular signal-regulated kinases), and PI_3K (phosphatidilinositil 3-kinase) attenuate carrageenin-induced peripheral hyperalgesia (Svensson et al., 2003; Choi et al., 2010; Fitzsimmons et al., 2010), formalin-induced overt-pain (Pezet et al., 2008) and nerve lesion-induced neuropathic pain (Obata et al., 2004; Xu et al., 2007; Gao and Ji, 2010). Thus, consistent data support that spinal inhibition of MAP kinases and PI_3K reduce inflammatory and neuropathic pain.

In this sense, G-CSF also induces MAP kinases and PI_3K activation dependent effects. G-CSF induces survival and/or proliferation via MAP kinases (p38, JNK and ERK) and/or PI_3K activation dependent pathways (Rausch and Marshall, 1997, 1999; Dong and Larner, 2000; Hunter and Avalos, 2000; Kendrick et al., 2004). Furthermore, anti-G-CSF antibody inhibits cancer-induced pain and spinal activation of ERK (Schweizerhof et al., 2009). However, it is not known whether peripheral administration of G-CSF induces pain via spinal activation of p38, ERK and JNK, and/or PI_3K. Therefore, we evaluated the spinal mechanisms involved in G-CSF injection-induced hyperalgesia in mice focusing on the participation of MAP kinases and PI_3K.

2. Materials and methods

2.1. Animals

The experiments were performed on male Swiss mice (20–25 g, Universidade Estadual de Londrina, Londrina, PR, Brazil) housed in standard clear plastic cages (five per cage) with free access to food and water. All behavioral testing was performed between 9:00 am and 5:00 pm in a temperature-controlled room. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina. All efforts were made to minimize the number of animals used and their suffering. It is noteworthy that different experimenters prepared the solutions, made the administrations and performed the evaluation of overt pain-like behavior, mechanical and thermal hyperalgesia.

2.2. Electronic pressure–meter test for mice

Mechanical hyperalgesia was tested in mice as previously reported (Cunha et al., 2004). Briefly, in a quiet room, mice were placed in acrylic cages ($12 \times 10 \times 17$ cm) with wire grid floors, 15–30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer (electronic von Frey anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) adapted with a 0.5 mm^2 contact area polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hind paw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements (indicated time points) after stimulus. The basal mechanical withdrawal threshold was 8.8 ± 0.1 g (mean ± SEM of 63 groups, 5 mice per group) before injection of stimulus or vehicle. There was no difference of basal mechanical withdrawal thresholds between groups in the same experiment.

2.3. Overt pain-like behavior evaluation

Mice were placed in clear glass compartments and the number of paw flinches and time spent licking the paw were determined during 30 min after i.pl. injection of saline (25 µl), G-CSF (100 ng/paw) or formalin 1.5% (25 µl). Total counts were presented at 5 min intervals (Valerio et al., 2009).

2.4. Hot plate test

Mice were placed in a 10 cm-wide glass cylinder on a hot plate (Insight, Ribeirão Preto, SP, Brazil) maintained at 55 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 5–9 s. The latency was also evaluated 30 and 60 min after test compound administration. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cut-off) was set at 30 s to avoid tissue damage (Valerio et al., 2007).

2.5. Intrathecal (i.t.) drug administration

The i.t. injections were performed under light halothane anesthesia (1–2%). The dorsal fur of each mouse was shaved, the spinal column was arched, and a 29-gauge needle was directly inserted into the subarachnoid space, between the L4 and L5 vertebrae (Mestre et al., 1994). Correct i.t. positioning of the needle tip was confirmed by manifestation of a characteristic tail flick response. A 5 µl volume containing the test agent was slowly injected. Note that drugs delivered to the subarachnoidal space by i.t. injection can diffuse into the CSF, which bathes the spinal cord, the dorsal roots, and part of dorsal root ganglion (Funez et al., 2008).

2.6. Drugs

Drugs were obtained from the following sources: formalin (1.5%, 25 µl i.pl.) from Merk (Darmstadt, Germany), G-CSF (Granulokine, filgrastin, recombinant human G-CSF, 30–300 ng/paw) from Hoffmann La-Roche (Basileia, Swiss), morphine sulphate (2–12 µg/paw) from Cristalia (São Paulo, Brazil), naloxone hydrochloride (1 mg/Kg), PD98059 (1–10 µg/intrathecal [i.t.]), SB202190 (1–10 µg/i.t.), SP600125 (1–10 µg/i.t.), and wortmanin (0.3–3 µg/i.t.) were obtained from Sigma-Aldrich (St Louis, MO, USA). G-CSF, morphine, naloxone and formalin were dissolved in saline, and all other compounds were dissolved in 20% DMSO in saline.

2.7. Statistical analysis

Results are presented as means ± s.e.m. of measurements made on 5 animals in each group. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the stimulus injection. The analyzed factors were treatments, time and time *versus* treatment interaction. When there was a significant time *versus* treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the hyperalgesic responses were measured once after the stimulus injection, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Additionally, comparative statistical analysis between two groups were done using *t* test. Statistical differences were considered to be significant at $P<0.05$.

3. Results

3.1. Intraplantar (i.pl. — subcutaneous injection in the paw) injection of G-CSF induces mechanical hyperalgesia

Saline (25 μl) or G-CSF (30–300 ng/paw) was injected via i.pl. route, and mechanical hyperalgesia was evaluated after 1–48 h with electronic pressure–meter test (Fig. 1). All doses of G-CSF tested induced significant mechanical hyperalgesia 1–7 h after administration, but only 100 and 300 ng of G-CSF induced significant hyperalgesia until 24 h, which decreased to control levels thereafter (48 h). One hundred and 300 ng of G-CSF induced significant hyperalgesia compared to the dose of 30 ng of G-CSF 7 and 24 h after stimulus, and 300 ng of G-CSF also induced significant hyperalgesia compared to 30 ng of G-CSF 5 h after stimulus. There was no statistical difference between 100 and 300 ng of G-CSF. Therefore, the G-CSF dose of 100 ng was chosen for the next experiments.

3.2. Morphine treatment inhibits G-CSF-induced hyperalgesia

Mice were treated with morphine (2, 6, and 12 μg/paw) or saline (20 μl) 4 h after i.pl. injection of G-CSF (Fig. 2A). Morphine dose-dependently inhibited G-CSF-induced hyperalgesia at 5 h after stimulus injection (Fig. 2A). There was a tendency of reduction of G-CSF-induced hyperalgesia by 2 μg/paw of morphine, although not significant. On the other hand, the doses of 6 and 12 μg/paw of morphine significantly inhibited G-CSF-induced hyperalgesia, and their effect was also significantly different of the dose of 2 μg of morphine. Corroborating the specificity of morphine inhibition, the treatment with naloxone (1 mg/kg, i.p., 1 h before morphine) significantly prevented morphine (6 μg/paw) inhibition of G-CSF-induced hyperalgesia at 5 h (Fig. 2B). The efficacy of 6 μg of morphine was local since had no effect on G-CSF-induced hyperalgesia when administrated in the contra-lateral paw (Fig. 2C). The effect of morphine was evaluated at one time point because at the local doses tested, its effect lasts for approximately 1 h (Verri et al., 2006, 2008a).

3.3. G-CSF administration does not induce overt pain-like behavior or thermal hyperalgesia

Mice received i.pl. injection of saline (25 μl), G-CSF or formalin 1.5% (25 μl), and the number of paw flinching (Fig. 3A) and time spent licking (Fig. 3B) were evaluated during 30 min at 5 min intervals. G-CSF did not induce significant paw flinching or licking compared to saline group (Fig. 3A and B). Additionally, other spontaneous nociceptive behaviors such as paw lifting or guarding were not observed (data not shown). On the other hand, the positive control

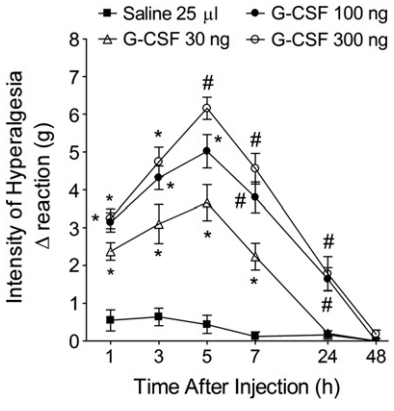

Fig. 1. Intraplantar injection of G-CSF induces mechanical hyperalgesia. Saline (25 μl) or G-CSF (30–300 ng/paw) was injected via intraplantar (i.pl.) route in mice and mechanical hyperalgesia was evaluated after 1–48 h with electronic pressure–meter test. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to the saline group, and # $P<0.05$ compared to the 30 ng/paw dose of G-CSF. (Two-way ANOVA, and one-way ANOVA followed by Tukey's t-test). The baseline mean values were: saline (8.3 ± 0.5), G-CSF 30 ng (8.5 ± 0.5), G-CSF 100 ng (8.8 ± 0.2), and G-CSF 300 ng (8.6 ± 0.4).

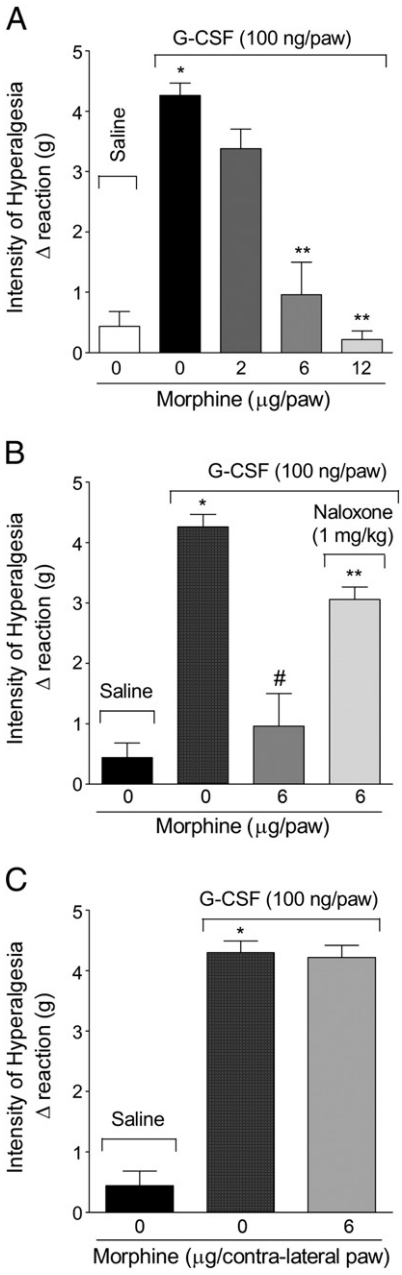

Fig. 2. Morphine treatment inhibits G-CSF-induced hyperalgesia. Panel A: Mice received i.pl. injection of G-CSF (100 ng) or vehicle (20 μl of saline), and after 4 h were treated with morphine (2, 6, and 12 μg/paw) or vehicle (20 μl of saline). Panel B: Mice received i.pl. injection of G-CSF (100 ng), and after 3 h were treated with naloxone (1 mg/kg, i.p. route) or vehicle (200 μl of saline). After additional 1 h, mice received morphine (6 μg/paw) treatment. Mechanical hyperalgesia was evaluated 5 h after G-CSF administration with electronic pressure meter. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to the saline group, # $P<0.05$ compared to G-CSF control group (Panel B only), and ** $P<0.05$ compared to G-CSF control group and the lower dose of morphine (2 μg/paw) (One-way ANOVA followed by Tukey's t-test). The baseline mean values were: Panel A — saline (9.3 ± 0.5), G-CSF (8.6 ± 0.4), G-CSF + morphine 2 (8.5 ± 0.3), G-CSF + morphine 6 (9.2 ± 0.6), and G-CSF + morphine 12 (9.7 ± 0.5); Panel B — saline (9.3 ± 0.5), G-CSF (8.6 ± 0.4), G-CSF + morphine (9.1 ± 0.6), and G-CSF + morphine + naloxone (8.3 ± 0.5); Panel C — saline (8.2 ± 0.2), G-CSF (8.0 ± 0.5), G-CSF + morphine contra-lateral paw (8.1 ± 0.4).

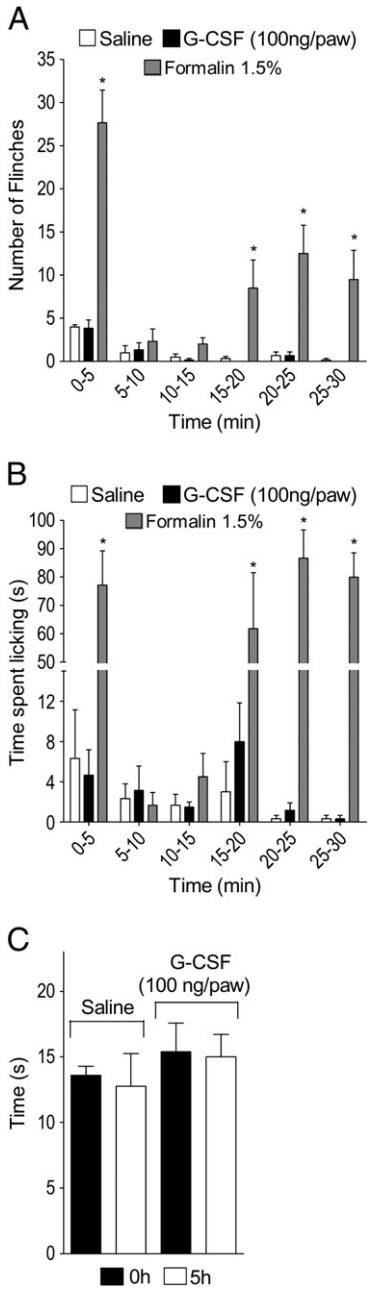

Fig. 3. G-CSF administration does not induce overt pain-like behavior or thermal hyperalgesia. Panels A–B: Total number of flinch (Panel A) and time spent licking (Panel B) were evaluated during 30 min after i.pl. injection of saline (25 μl), G-CSF (100 ng) or formalin 1.5% (25 μl) in mice. Panel C: The thermal nociceptive threshold was evaluated before and 5 h after i.pl. injection of saline (25 μl) or G-CSF (100 ng) with the hot plate test in mice. Bars represent means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. No statistical differences were detected in panels A–B (one-way ANOVA followed by Tukey's t-test) and panel C (t-test).

group that received i.pl. injection of formalin presented significant paw flinching and licking compared to saline and G-CSF between 0–5, 15–20, 20–25 and 25–30 min (Fig. 3A and B). The formalin-induced overt pain-like behavior was consistent with the model since presented two phases (Valerio et al., 2009). In another set of experiments, the thermal nociceptive threshold was evaluated before and 5 h after i.pl. injection of saline (25 μl) or G-CSF with the hot plate test in mice (Fig. 3C). G-CSF did not alter the thermal threshold of mice in the hot plate test.

3.4. Role of spinal ERK activation in G-CSF-induced hyperalgesia

Mice were treated intrathecally (i.t.) with the MEK1/2 inhibitor (prevents ERK1/2 activation) PD98059 (1–10 μg) or vehicle (5 μl of 20% DMSO in saline) 30 min before i.pl. G-CSF stimulus, and mechanical hyperalgesia was evaluated in the ipsilateral (Fig. 4A) and contra-lateral (Fig. 4B) paws to G-CSF stimulus. These dose ranges and vehicle concentration were chosen based on previous studies (Zhuang et al., 2004, 2005). The dose of 1 μg of PD98059 did not alter G-CSF-induced hyperalgesia (Fig. 4A). On the other hand, the dose of 3 μg of PD98059 inhibited G-CSF-induced hyperalgesia 5 h after stimulus (Fig. 4A). The dose of 10 μg of PD98059 significantly inhibited G-CSF hyperalgesia 1–7 h compared to vehicle i.t. + G-CSF, and 3–7 h compared to PD98059 1 μg i.t. + G-CSF groups (Fig. 4A). The

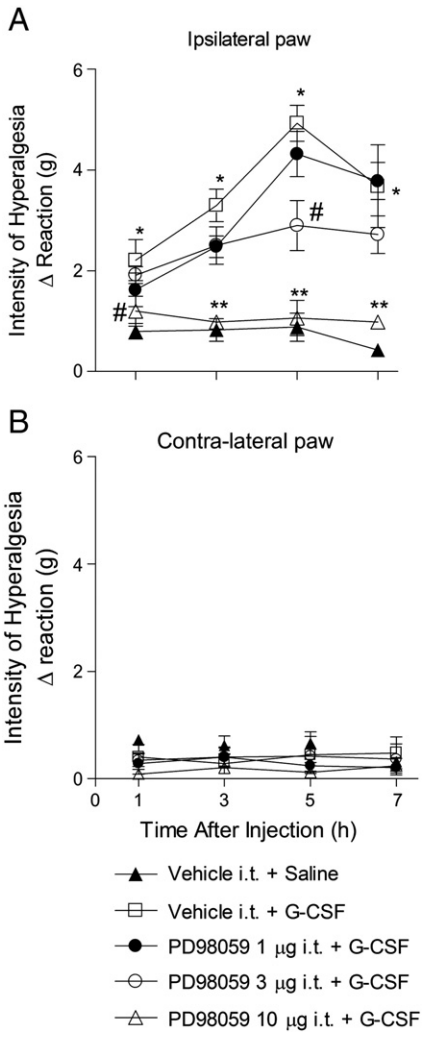

Fig. 4. Role of spinal ERK in G-CSF-induced hyperalgesia. Mice were treated with PD98059 (1–10 μg) or vehicle (2% DMSO in saline) via intrathecal (i.t.) route 30 min before i.pl. injection with G-CSF (100 ng/paw). Mechanical hyperalgesia was evaluated in the ipsilateral (Panel A) and contra-lateral (Panel B) paws to G-CSF injection after 1–7 h with electronic pressure meter test. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to vehicle i.t. + saline group, # $P<0.05$ compared to vehicle i.t. + G-CSF group, and ** $P<0.05$ compared to the lower dose of inhibitor tested (Two-way ANOVA, and one-way ANOVA followed by Tukey's t-test). The baseline mean values were: Panel A — vehicle i.t. + saline (8.8 ± 0.2), vehicle i.t. + G-CSF (8.9 ± 0.3), PD98059 1 μg i.t. + G-CSF (8.4 ± 0.2), PD98059 3 μg i.t. + G-CSF (8.8 ± 0.2), and PD98059 10 μg i.t. + G-CSF (8.7 ± 0.6); Panel B — vehicle i.t. + saline (8.6 ± 0.6), vehicle i.t. + G-CSF (8.4 ± 0.2), PD98059 1 μg i.t. + G-CSF (8.6 ± 0.4), PD98059 3 μg i.t. + G-CSF (8.5 ± 0.2), and PD98059 10 μg i.t. + G-CSF (8.7 ± 0.2).

PD98059 i.t. treatment did not alter the mechanical hyperalgesia in the contra-lateral paw (Fig. 4B).

3.5. Role of spinal JNK in G-CSF-induced hyperalgesia

Mice were treated i.t. with the JNK inhibitor SP600125 (1–10 μg) or vehicle (5 μl of 20% DMSO in saline) 30 min before i.pl. G-CSF stimulus, and mechanical hyperalgesia was evaluated in the ipsilateral (Fig. 5A) and contra-lateral (Fig. 5B) paws to G-CSF stimulus. These dose ranges and vehicle concentration were chosen based on previous studies (Doya et al., 2005). The dose of 1 μg of SP600125 did not alter G-CSF-induced hyperalgesia (Fig. 5A). On the other hand, the dose of 3 μg of SP600125 inhibited G-CSF-induced hyperalgesia 3 and 5 h after stimulus injection (Fig. 5A). The dose of 10 μg of SP600125 significantly inhibited G-CSF hyperalgesia compared to vehicle i.t. + G-CSF and SP600125 1 μg i.t. + G-CSF groups 3–7 h after stimulus injection (Fig. 5A). The SP600125 i.t. treatment did not alter the mechanical hyperalgesia in the contra-lateral paw (Fig. 5B).

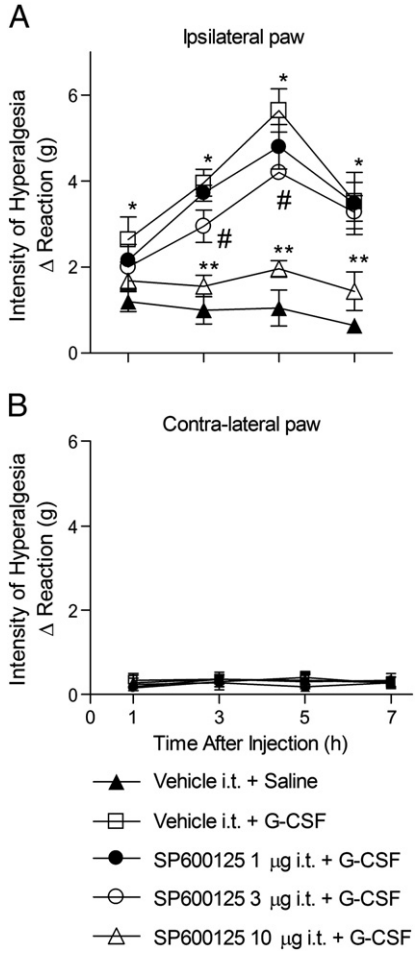

Fig. 5. Role of spinal JNK in G-CSF-induced hyperalgesia. Mice were treated with SP600125 (1–10 μg) or vehicle (2% DMSO in saline) via intrathecal (i.t.) route 30 min before i.pl. injection with G-CSF (100 ng/paw). Mechanical hyperalgesia was evaluated in the ipsilateral (Panel A) and contra-lateral (Panel B) paws to G-CSF injection after 1–7 h with electronic pressure meter test. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to vehicle i.t. + saline group, # $P<0.05$ compared to vehicle i.t. + G-CSF group, and ** $P<0.05$ compared to the lower dose of inhibitor tested (Two-way ANOVA, and one-way ANOVA followed by Tukey's t-test). The baseline mean values were: Panel A — vehicle i.t. + saline (10.5 ± 0.2), vehicle i.t. + G-CSF (10.3 ± 0.2), SP600125 1 μg i.t. + G-CSF (10.3 ± 0.4), SP600125 3 μg i.t. + G-CSF (10.5 ± 0.6), and SP600125 10 μg i.t. + G-CSF (10.4 ± 0.2); Panel B — vehicle i.t. + saline (10.5 ± 0.2), vehicle i.t. + G-CSF (10.3 ± 0.1), SP600125 1 μg i.t. + G-CSF (9.7 ± 0.7), SP600125 3 μg i.t. + G-CSF (10.1 ± 0.3), and SP600125 10 μg i.t. + G-CSF (10.2 ± 0.2).

3.6. Role of spinal p38 in G-CSF-induced hyperalgesia

Mice were treated i.t. with the p38 inhibitor SB202190 (1–10 μg) or vehicle (5 μl of 20% DMSO in saline) 30 min before i.pl. G-CSF stimulus, and mechanical hyperalgesia was evaluated in the ipsilateral (Fig. 6A) and contra-lateral (Fig. 6B) paws to G-CSF stimulus. These dose ranges and vehicle concentration were chosen based on previous studies (Chen et al., 2009). The dose of 1 μg of SB202190 did alter G-CSF-induced hyperalgesia (Fig. 6A). The dose of 3 μg of SB202190 inhibited G-CSF-induced hyperalgesia 5 h after stimulus (Fig. 6A). The dose of 10 μg of SB202190 significantly inhibited G-CSF hyperalgesia 3–7 h compared to vehicle i.t. + G-CSG group, and 3–5 h compared to the dose of 1 μg of SB202190 (Fig. 6A). The SB202190 i.t. treatment did not alter the mechanical hyperalgesia in the contra-lateral paw (Fig. 5B).

3.7. Role of spinal PI_3K in G-CSF-induced hyperalgesia

Mice were treated i.t. with the PI_3K inhibitor wortmanin (0.3–3 μg) or vehicle (5 μl of 20% DMSO in saline) 30 min before i.pl. G-CSF

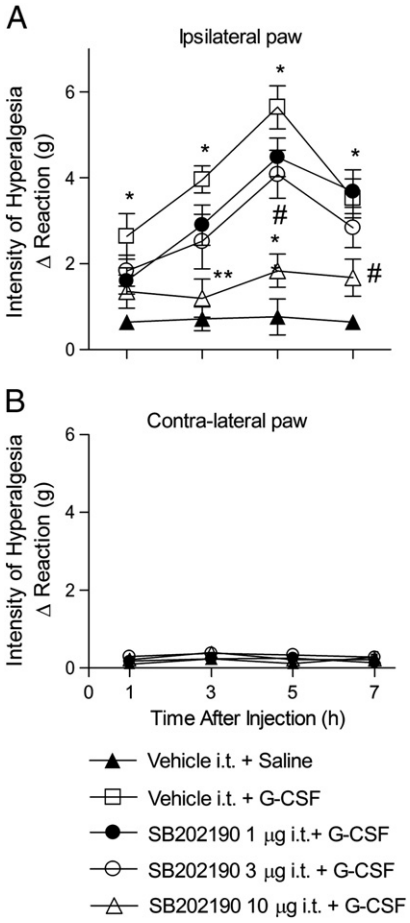

Fig. 6. Role of spinal p38 in G-CSF-induced hyperalgesia. Mice were treated with SB202190 (1–10 μg) or vehicle (2% DMSO in saline) via intrathecal route 30 min before i.pl. injection with G-CSF (100 ng/paw). Mechanical hyperalgesia was evaluated in the ipsilateral (Panel A) and contra-lateral (Panel B) paws to G-CSF injection after 1–7 h with electronic pressure meter test. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to vehicle i.t. + saline group, # $P<0.05$ compared to vehicle i.t. + G-CSF group, and ** $P<0.05$ compared to the lower dose of inhibitor tested (Two-way ANOVA, and one-way ANOVA followed by Tukey's t-test). The baseline mean values were: Panel A — vehicle i.t. + saline (7.9 ± 0.3), vehicle i.t. + G-CSF (8.5 ± 0.2), SB202190 1 μg i.t. + G-CSF (8.4 ± 0.2), SB202190 3 μg i.t. + G-CSF (8.8 ± 0.2), and SB202190 10 μg i.t. + G-CSF (8.6 ± 0.5); Panel B — vehicle i.t. + saline (7.8 ± 0.2), vehicle i.t. + G-CSF (8.4 ± 0.3), SB202190 1 μg i.t. + G-CSF (8.6 ± 0.2), SB202190 3 μg i.t. + G-CSF (8.7 ± 0.2), and SB202190 10 μg i.t. + G-CSF (8.5 ± 0.4).

stimulus, and mechanical hyperalgesia was evaluated in the ipsilateral (Fig. 7A) and contra-lateral (Fig. 7B) paws to G-CSF stimulus. These dose ranges and vehicle concentration were chosen based on previous studies (Zhuang et al., 2004; Xu et al., 2007). The dose of 0.3 μg of wortmanin did not alter G-CSF-induced hyperalgesia (Fig. 7A). On the other hand, 1 and 3 μg of wortmanin significantly inhibited the hyperalgesia compared to vehicle i.t. + G-CSF and 0.3 μg i.t. + G-CSF groups 3–7 h after stimulus (Fig. 7A). The dose of 3 μg of wortmanin inhibitory effect was also significant 1 h after stimulus compared to vehicle i.t. + G-CSF group (Fig. 7A). The wortmanin i.t. treatment did not alter the mechanical hyperalgesia in the contra-lateral paw (Fig. 7B). Five h after G-CSF stimulus there were significant differences between the doses of MAP kinases and PI_3K inhibitors (Figs. 4A, 5A, 6A and 7A), which is also the peak of G-CSF-induced mechanical hyperalgesia (Fig. 1). Therefore, this time point was used for the next experiment.

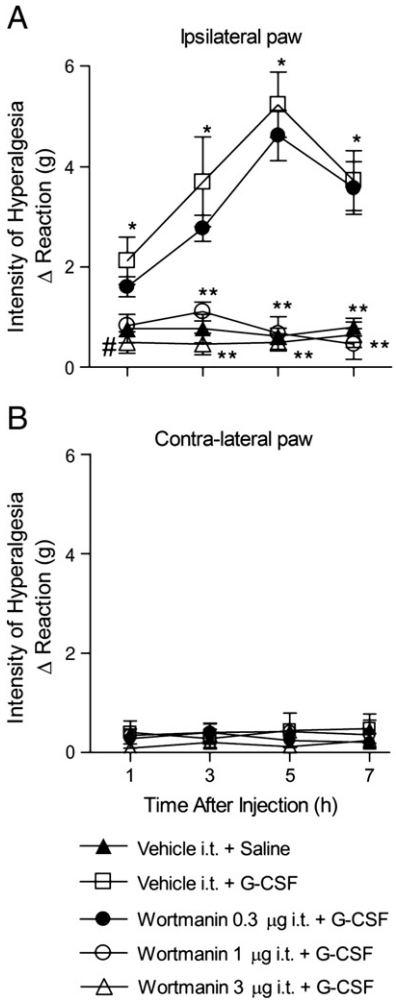

Fig. 7. Role of spinal PI_3K in G-CSF-induced hyperalgesia. Mice were treated with wortmanin (0.3–3 μg) or vehicle (2% DMSO in saline) via intrathecal route 30 min before i.pl. injection with G-CSF (100 ng/paw). Mechanical hyperalgesia was evaluated in the ipsilateral (Panel A) and contra-lateral (Panel B) paws to G-CSF injection after 1–7 h with electronic pressure meter test. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to vehicle i.t. + saline group, # $P<0.05$ compared to vehicle i.t. + G-CSF group, and ** $P<0.05$ compared to the lower dose of inhibitor tested (Two-way ANOVA, and one-way ANOVA followed by Tukey's t-test). The baseline mean values were: Panel A — vehicle i.t. + saline (7.8 ± 0.6), vehicle i.t. + G-CSF (8.7 ± 0.2), wortmanin 0.3 μg i.t. + G-CSF (8.6 ± 0.5), wortmanin 1 μg i.t. + G-CSF (8.6 ± 0.4), and wortmanin 3 μg i.t. + G-CSF (8.5 ± 0.2); Panel B — vehicle i.t. + saline (7.9 ± 0.3), vehicle i.t. + G-CSF (8.4 ± 0.2), wortmanin 0.3 μg i.t. + G-CSF (8.7 ± 0.4), wortmanin 1 μg i.t. + G-CSF (8.3 ± 0.5), and wortmanin 3 μg i.t. + G-CSF (8.6 ± 0.4).

3.8. Combined Treatment with MAP kinases and PI_3K inhibitors at doses that are ineffective as single treatment reduces G-CSF-induced hyperalgesia

Mice received single i.t. treatment with PD98059 (1 μg), SP600125 (1 μg), SB202190 (1 μg) or wortmanin (0.3 μg), or co-treatment with 1 μg of each MAP kinase inhibitors (PD98059, SP600125, SB202190) plus 0.3 μg of wortmanin in a single injection or vehicle (5 μl of 2% DMSO in saline) 30 min before G-CSF stimulus (Fig. 8). G-CSF induced significant hyperalgesia 5 h after injection, which was unaffected by single treatment with PD98059, SP600125, SB202190 or wortmanin. On the other hand, the combination of those inhibitors at doses that were ineffective as single treatment significantly inhibited G-CSF-induced hyperalgesia (Fig. 8).

4. Discussion

Granulocyte-colony stimulating factor (G-CSF) is a current therapy to increase neutrophil counts in peripheral blood of patients that underwent chemotherapy or radiotherapy for cancer treatment. The G-CSF therapy is well tolerated, but some side effects such as abdominal pain, bone pain and muscle-skeletal pain limit its applicability (Granulokine®, Filgrastim package insert; Battiwalla and McCarthy, 2009). In the present study, it was demonstrated that G-CSF-induced mechanical hyperalgesia in mice depends on spinal activation of ERK, JNK, p38 and PI_3K acting in synergy/sequence. Furthermore, the treatment with a dose of morphine with peripheral action also reduced G-CSF-induced hyperalgesia.

The intraplantar (subcutaneous plantar) injection of G-CSF induced hyperalgesia in mice at a dose equivalent to the recommended dose for humans of 5 μg/kg per day (Granulokine®, Filgrastim package insert). In humans, opioid treatment is a usual and efficient approach to inhibit G-CSF therapy-induced pain. In agreement, G-CSF-induced mechanical hyperalgesia in mice was reduced by morphine treatment, and this effect of morphine was inhibited by naloxone treatment. Thus, the response induced by G-CSF in mice seems to reflect what is seen in humans. It is important to note that patients may undergo G-CSF therapy for many weeks, and chronic use of opioids raise possible issues such as side effects, tolerance and addiction (Devulder et al., 2009). In this sense, our study also suggests that it is worthy evaluating whether treatment with peripherally acting opioids is a conceivable approach to control G-CSF therapy-induced pain since morphine was effective at peripherally acting doses (e.g. ipsilateral but not contra-lateral treatment with morphine inhibited G-CSF hyperalgesia).

G-CSF can directly activate nociceptors since they express G-CSF receptor (G-CSFR) (Schweizerhof et al., 2009). However, it was not detected overt pain-like behavior by the hyperalgesic dose of 100 ng of G-CSF. This result is consistent with the effect of other cytokines that do not induced overt pain at hyperalgesic doses (Verri et al., 2008b). G-CSF did not induce thermal hyperalgesia, which suggests disagreement with previous evidence (Schweizerhof et al., 2009). Nevertheless, the use of different thermal tests involving different structure/mechanisms such as spinal (Hargreaves plantar test) *versus* supra-spinal (hot plate test) mechanisms (Le Bars et al., 2001) might explain these opposing results (Schweizerhof et al., 2009 and present data, respectively). Therefore, G-CSF seems to activate spinal rather than supra-spinal mechanisms.

A common mechanism of inflammatory and neuropathic pain is the spinal activation of MAP kinases (ERK, JNK and p38) and PI_3K (Svensson et al., 2003; Ji and Strichartz, 2004; Obata et al., 2004; Xu et al., 2007; Pezet et al., 2008; Choi et al., 2010; Fitzsimmons et al., 2010; Gao and Ji, 2010). The activation of spinal MAP kinases and PI_3K contribute to hyperalgesia by modulating ion channels, increasing the production of cytokines and other mediators and their receptors, and therefore, inducing the sensitization of nociceptors (Ji & Strichartz, 2004; Gao and Ji, 2010).

Interestingly, G-CSF also activates those kinases in other systems (Rausch and Marshall, 1997, 1999; Dong and Larner, 2000; Hunter and Avalos, 2000; Kendrick et al., 2004). In agreement, inhibition of p38, ERK, JNK, and PI_3K diminished G-CSF-induced hyperalgesia in a dose-dependent manner. Importantly, none of the intrathecal treatments with kinase inhibitors altered the mechanical hyperalgesia in the contra-lateral paw to G-CSF stimulus, indicating that PD98059, SB600125, SB202190 and wortmanin do not alter the basal mechanical threshold of normal tissue. Furthermore, the higher doses of p38, ERK, JNK, and PI_3K inhibitors abolished G-CSF-induced hyperalgesia, which could be explained by a sequential or synergic role of spinal p38, ERK, JNK, and PI_3K in G-CSF-induced hyperalgesia. In fact, the combined treatment with MAP kinases and PI_3K inhibitors, at doses that are ineffective as single treatment, significantly reduced G-CSF hyperalgesia, therefore, indicating that these pathways act in sequence/synergy. Indicating a sequential pathway and the interaction of PI_3K and MAP kinases, PI_3K activates ERK in primary sensory neurons inducing heat hyperalgesia (Zhuang et al., 2004), and PI_3K activates p38 inducing cellular chemotaxis (Shahabuddin et al., 2006). MAP kinases present a different interaction among them compared to PI_3K/MAP kinases relationship since they can act in a co-dependent manner to activate transcription factors such as activating protein-1 (AP-1) as known for JNK and ERK (Kim and Iwao, 2003). On the other hand, p38, ERK and JNK do not activate each other. Corroborating, ERK and p38 inhibitors do not affect G-CSF-induced JNK activation in a model of cell proliferation (Rausch and Marshall, 1997). Thus, the combined treatment with MAP kinases and PI_3K inhibitors could allow reduced doses of such inhibitors (1 μg of each MAP kinase inhibitor plus 0.3 μg of PI_3K inhibitor) compared to single drug treatment (10 μg of a single MAP kinase inhibitor or 1–3 μg of PI_3K inhibitor).

In addition to the present data, it was recently demonstrated the endogenous role of G-CSF in a mice model of bone cancer pain (Schweizerhof et al., 2009). The injection of pancreatic adenocarcinoma induces an increase of G-CSF production in the paw skin concomitantly with expression of G-CSFR by sensory dorsal root ganglion (DRG) neurons suggesting possible direct activation of nociceptors by G-CSF (Schweizerhof et al., 2009). Furthermore, the treatment with anti-G-CSFR antibodies reduces tumor-induced mechanical hyperalgesia, demonstrating the G-CSF endogenous role in this model of cancer pain (Schweizerhof et al., 2009). G-CSF induces ERK activation in dorsal root ganglion neurons *in vitro* in a PI_3K and MEK inhibitors (LY294002 and PD98059, respectively) sensitive manner, suggesting that G-CSF-induced ERK phosphorilation depends on prior PI_3K activation (Schweizerhof et al., 2009). This result supports our hypothesis described above in which MAP kinases and PI_3K pathways are interconnected and sequentially/synergistically mediate G-CSF hyperalgesia. Corroborating a role for G-CSF-triggered mechanisms in tumor-induced hyperalgesia, PD98059 treatment also inhibits carcinoma-induced hyperalgesia (Schweizerhof et al., 2009). Thus, Schweizerhof et al. (2009) demonstrated that endogenous G-CSF mediates tumor-induced hyperalgesia in a PI_3K/ERK-dependent pathway. Nevertheless, it was not addressed whether the hyperalgesia induced by G-CSF administration could be diminished by MAP kinases or PI_3K inhibitors, which is an experimental condition that could shed light in the pain mechanisms triggered by G-CSF therapy as demonstrated herein.

The present study confirmed that G-CSF induces mechanical hyperalgesia, and advanced by demonstrating an *in vivo* role of spinal MAP kinases (ERK, JNK and p38) and PI_3K in G-CSF-induced mechanical hyperalgesia in mice.

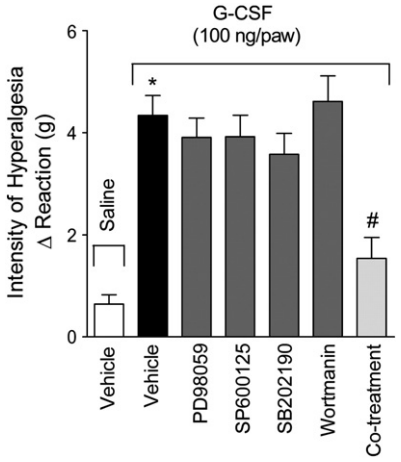

Fig. 8. Combined Treatment with MAP kinases and PI_3K inhibitors at doses that are ineffective as single treatment reduces G-CSF-induced hyperalgesia. Mice received single i.t. treatment with PD98059 (1 μg), SP600125 (1 μg), SB202190 (1 μg) or wortmanin (0.3 μg), or co-treatment with 1 μg of each MAP kinase inhibitor (PD98059, SP600125, and SB202190) plus 0.3 μg of wortmanin or vehicle (2% DMSO in saline) 30 min before i.pl. injection with G-CSF (100 ng/paw). Mechanical hyperalgesia was evaluated 5 h after G-CSF administration with electronic pressure meter. Results are presented as means ± s.e.m. of 5 mice per group, and are representative of 2 separated experiments. * $P<0.05$ compared to vehicle i.t. + saline group, # $P<0.05$ compared to vehicle + G-CSF control group, and inhibitors alone groups (One-way ANOVA followed by Tukey's t-test). The baseline mean values were: vehicle i.t. + saline (8.0 ± 0.6), vehicle i.t. + G-CSF (8.3 ± 0.2), PD98059 i.t. + G-CSF (7.9 ± 0.2), SP600125 i.t. + G-CSF (8.2 ± 0.1), SB202190 i.t. + G-CSF (8.0 ± 0.3), wortmanin i.t. + G-CSF (8.2 ± 0.5), and co-treatment (8.1 ± 0.3).

Acknowledgments

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