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p38 stress-activated protein kinase inhibitor reverses bradykinin B_1 receptor-mediated component of inflammatory hyperalgesia

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Abstract

The effects of a p38 stress-activated protein kinase inhibitor, 4-(4-fluorophenyl)-2-(-4-methylsulfonylphenyl)-5-(4-pyridynyl) imidazole (SB203580), were evaluated in a rat model of inflammatory hyperalgesia. Oral, but not intrathecal, administration of SB203580 significantly reversed inflammatory mechanical hyperalgesia induced by injection of complete Freund's adjuvant into the hindpaw. SB203580 did not, however, affect the increased levels of interleukin-1β and cyclo-oxygenase 2 protein observed in the hindpaw following complete Freund's adjuvant injection. Intraplantar injection of interleukin-1β into the hindpaw elicited mechanical hyperalgesia in the ipsilateral paw, as well as in the contralateral paw, following intraplantar injection of the bradykinin B_1 receptor agonist des-Arg^9-bradykinin. Oral administration of SB203580 1 h prior to interleukin-1β administration prevented the development of hyperalgesia in the ipslateral paw and the contralateral bradykinin B_1 receptor-mediated hyperalgesia. In addition, following interleukin-1β injection into the ipsilateral paw, co-administration of SB203580 with des-Arg^9-bradykinin into the contralateral paw inhibited the bradykinin B_1 receptor-mediated hyperalgesia.

In human embryonic kidney 293 cells expressing the human bradykinin B_1 receptor, its agonist des-Arg^{10}-kallidin produced a rapid phosphorylation of endogenous p38 stress-activated protein kinase. Our data suggest that p38 stress-activated protein kinase is involved in the development of inflammatory hyperalgesia in the rat, and that its pro-inflammatory effects involve the induction of the bradykinin B_1 receptor as well as functioning as its downstream effector.

Keywords: Inflammatory hyperalgesia; p38 stress-activated protein kinase; Interleukin-1β; Bradykinin B_1 receptor; SB203580

1. Introduction

Cytokines are key modulators in inflammatory processes following infection and tissue injury (see Arai et al., 1990 for review). Considerable evidence now indicates a key role for pro-inflammatory cytokines, such as tumour necrosis factor-α and interleukin-1, in the development of hyperalgesia associated with inflammation. For example, peripheral or central administration of interleukin-1β and tumour necrosis factor-α has been shown to produce hyperalgesia in rats (Ferreira et al., 1988; Cunha et al., 1992; Watkins et al., 1994, 1995, Davis and Perkins, 1994), while complete Freund's adjuvant-induced hyperalgesia in the rat is associated with increased tissue levels of both cytokines (Safieh-Garabedian et al., 1995; Woolf et al., 1997). The role of cytokines in inflammatory hyperalgesia may be via a direct action on sensory nerve terminals (Opree and Kress, 2000), or via the release of other mediators. A notable consequence of cytokine signalling is the induction of cyclo-oxygenase 2 enzyme (Maier et al., 1990), leading to the production of pro-inflammatory prostanoids such as prostaglandin E_2, which is known to be a key mediator in inflammatory hyperalgesia (Chan et al., 1995; Mitchell et al., 1995; Hay et al., 1997).

Another significant component of inflammatory hyperalgesia is the activation of bradykinin B_1 receptors (Dray and Perkins, 1993). Bradykinin B_1 receptors are generally not expressed under normal conditions, but are induced during inflammation or tissue damage (Marceau et al., 1998). In a number of different cell types, interleukin-1β has been shown to up-regulate bradykinin B_1 receptor expression and enhance responses to its ligands, des-Arg^9-bradykinin and des-Arg^{10}-kallidin (Lerner and Mod-

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eer, 1991; Galizzi et al., 1994; Ni et al., 1998; Tsukagoshi et al., 1999; Haddad et al., 2000). A similar induction of bradykinin B_1 receptor-mediated responses has been described in vivo. Thus, inflammatory mechanical and thermal hyperalgesia are inhibited by bradykinin B_1 receptor antagonists, and the bradykinin B_1 receptor agonist des-Arg^9-bradykinin produces hyperalgesia following interleukin-1β administration (Perkins et al., 1993; Davis and Perkins, 1994; Perkins and Kelly, 1994).

A key component of the cytokine signal transduction pathway is the p38 stress-activated protein kinase family (Lee et al., 1994). Activation of this family leads to induction of many proteins central to inflammatory processes including further induction of cytokine secretion (Lee et al., 1994; Eder, 1997; Jackson et al., 1998). Inhibitors of p38 stress-activated protein kinase have been shown to be effective in a variety of models of inflammation, although there is little information on their activity in models of hyperalgesia. Here, we have examined the effects of a p38 stress-activated protein kinase inhibitor, 4-(4-fluorophenyl)-2-(4-methylsulfonylphenyl)-5-(4-pyridynyl) imidazole (SB203580) (Badger et al., 1996) on mechanical hyperalgesia, and the involvement of bradykinin B_1 receptor, as well as the production of cyclooxygenase 2 and inflammatory cytokines in models of inflammatory pain in the rat (Stein et al., 1988).

2. Materials and methods

All experiments were carried out according to Home Office (United Kingdom) guidelines and with approval of the Novartis Animal Welfare and Ethics Committee.

2.1. Complete Freund's adjuvant-induced mechanical hyperalgesia

Male Sprague–Dawley rats (200–250 g) received an intraplantar injection of 25 μl complete Freund's adjuvant into one hind paw. Mechanical hyperalgesia was assessed 24 h later using an analgesymeter (Ugo Basile, Milan) by measuring withdrawal thresholds to an increasing pressure stimulus placed onto the dorsal surface of the paw. The cut-off was set at 250 g and the end-point taken as paw withdrawal, vocalisation or overt struggling. Paw withdrawal thresholds were measured in both hindpaws prior to (naive) and 24 h following complete Freund's adjuvant injection (predose). SB203580 was then administered either orally or intrathecally and withdrawal thresholds measured up to 6 h later (postdose). The percentage reversal of hyperalgesia was calculated as follows:

$$\%\text{reversal} = \frac{\text{postdose threshold} - \text{predose threshold}}{\text{naive threshold} - \text{predose threshold}} \times 100$$

2.2. Bradykinin B_1 receptor-mediated hyperalgesia

Hyperalgesia was induced by interleukin-1β administration into the hindpaw. Mechanical withdrawal thresholds were measured in both hindpaws as described above prior to and 1 h following injection of interleukin-1β (10 ng) into the ipsilateral paw. The bradykinin B_1 receptor agonist des-Arg^9-bradykinin was then injected into the contralateral paw, and withdrawal thresholds were again measured up to 4 h later. The involvement of bradykinin B_1 receptors was confirmed by co-administering the selective antagonist [Leu^8] des-Arg^{9}bradykinin with des-Arg^9-bradykinin. The effect of SB203580 on the induction of bradykinin B_1 receptor-mediated hyperalgesia was examined by administering the inhibitor orally 1 h prior to interleukin-1β injection. In separate experiments, SB203580 was administered at the same time as des-Arg^9-bradykinin to test whether the p38 stress-activated protein kinase pathway is involved directly in the bradykinin B_1 receptor-mediated hyperalgesia.

2.3. Preparation of rat paw homogenates

Inflamed and non-inflamed hindpaws were removed from terminally anaesthetised rats treated with SB203580 or vehicle, and from naive, non-inflamed animals receiving no treatment. SB203580 or vehicle was administered orally to animals 24 h following complete Freund's adjuvant injection into one hindpaw. Animals were then killed by overdose of anaesthetic 30 min–6 h following drug administration, and both ipsilateral and contralateral hindpaws were then removed. Each paw was cut into small pieces, snap frozen in liquid nitrogen and then stored at −70°C. At the time of experiment, tissues were homogenised for 3×10 s in 6 ml of ice-cold lysis buffer (10 mM Tris pH 7.6, 5 mM ethylenediamine tetraacetic acid, 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 100 μM sodium vanadate, 1% Triton X-100, 1 mM phenylmethyl sulphonyl fluoride). The homogenates were left on ice for 1 h to maximise solubilisation of proteins and then centrifuged at 15,000 rpm at 4°C for 20 min. Supernatant fractions were clarified through 0.2-μm filters and protein concentration was measured by a detergent-compatible Bradford assay (Bio-Rad Laboratories). Supernatants were stored until use in aliquots at −70°C.

2.4. Measurement of interleukin-1β, interleukin-1α and tumour necrosis factor-α levels

Aliquots prepared from the complete Freund's adjuvant inflammatory model were evaluated by enzyme-linked immunosorbent assay for levels of interleukin-1β, interleukin-1α, and tumour necrosis factor-α according to manufacturer's instructions (Endogen, MA, USA). Each time point represents the mean of three samples from

separate animals, each evaluated in triplicate. Results are expressed as picogram cytokine per milligram of total protein.

2.5. Measurement of cyclo-oxygenase 2 levels

Levels of cyclo-oxygenase 2 protein in hindpaw tissue were determined by immunoblotting methods. Approximately 30 μg of total protein, prepared from hindpaws as described above, was loaded and resolved onto 8% denaturing electrophoresis gels. Proteins were electrophoretically transferred on to nitrocellulose membranes (Schleicher and Schuell), which were then incubated in a 5% solution of milk proteins in Tris-buffered saline containing Tween 20 (10 mM Tris, pH 8.0, 150 mM sodium chloride, 0.1% Tween 20) to block non-specific binding of antibodies. The membranes were incubated with goat anti-cyclo-oxygenase 2 (0.3 μg/ml; Santa Cruz Biotechnology, CA, USA) and mouse anti-α-tubulin (1:1000; Pierce and Warriner, UK) primary antibodies according to manufacturers recommendations followed by horse radish peroxidase conjugated anti-goat and anti-mouse secondary antibodies both at 1:3000 dilutions. The Western blots were developed by chemiluminescence using Supersignal West Pico chemiluminescent substrate (Pierce and Warriner) according to manufacturer's instructions. Protein levels were determined by measuring chemiluminescence using a Fluor-S Max multi-imager (Bio-Rad Laboratories). Cyclo-oxygenase 2 protein levels were normalised to those of an internal control, α-tubulin, in the same lane to control for variations in sample loading. To compare the effects of inflammation and drug treatment on cyclo-oxygenase 2 levels, the amount of this enzyme in samples from naive animals was arbitrarily set at 1, and all other samples were expressed relative to this.

2.6. Measurement of p38 stress-activated protein kinase phosphorylation levels

Bradykinin B_1 receptor-mediated p38 stress-activated protein kinase phosphorylation was examined in cells expressing the human bradykinin B_1 receptor. Human embryonic kidney 293 cells were transfected with an HB1-pcDNA3 construct containing the human bradykinin B_1 receptor sequence and receptor expressing clones isolated using G418 selection and a specific radioligand binding assay, as previously described (Jones et al., 1999). Cells were maintained in minimum essential medium with Earle's salts (Gibco) supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% nonessential amino acids and 10% myoclone foetal calf serum (Gibco), under G418 selection (Sigma or Gibco) added at 700 μg/ml, in a humidified atmosphere with 5% CO_2 at 37°C. The medium was replaced every 3–4 days.

For the p38 stress-activated protein kinase phosphorylation, assay cells were starved of serum for 16–18 h and then stimulated or mock-stimulated in fresh serum-free medium for 1–60 min with the human bradykinin B_1 receptor selective agonist des-Arg^{10}-kallidin (1 μM) at 37°C. To extract proteins, the cells were washed once in ice-cold phosphate-buffered saline and lysed in hot sample buffer. Lysates were then vortexed and centrifuged, and 10 μl of the supernatants were resolved on 10% sodium dodecyl sulphate polyacrylamide gels. Gels were processed for Western blotting as described in Section 2.5. The membranes were probed with 1:1000 dilution of a rabbit phospho-p38 stress-activated protein kinase specific antibody (New England Biolabs). Anti-rabbit-horse radish peroxidase secondary antibodies (Santa Cruz Biotechnology) were used at 1:2500 and the immunoblots were developed as described in Section 2.5. Membranes were reprobed with anti α-tubulin antibodies to normalise for variation in protein loading. To examine the effects of des-Arg^{10}-kallidin-mediated phosphorylation on p38 stress-activated protein kinase, the amount of phosphorylation observed in the absence of stimulation was arbitrarily set at 1, and all other samples were expressed relative to this.

2.7. Drugs

For the behavioural experiments, SB203580 (Alexis, UK) was dissolved in 0.5% dimethyl sulphoxide/1% tragacanth for oral administration, and 0.5% DMSO for intrathecal administration. Interleukin-1β (R&D Europe) was dissolved in phosphate buffered saline and 0.1% bovine serum albumin, and des-Arg^9-bradykinin (R&D Europe) and [Leu^8] des-Arg^9-bradykinin (R&D Europe) were both dissolved in saline. Drug or vehicle were administered in volumes of 0.5 ml for oral administration, 10 μl for intrathecal administration and 20 μl for intraplantar administration.

2.8. Data analysis

All behavioural experiments used six or eight animals per group. Statistical analysis was carried out on withdrawal threshold readings using analysis of variance, followed by post hoc analysis of the means using Tukey's honest squared difference test comparing drug-treated with vehicle-treated animals. Data for biochemical experiments were analysed using paired or unpaired *t*-test as appropriate. In all experiments, $P < 0.05$ was considered significant.

3. Results

3.1. Inflammatory hyperalgesia

Oral administration of SB203580 (0.25–25 mg/kg) produced a pronounced reversal of complete Freund's adjuvant-induced mechanical hyperalgesia (Fig. 1). The

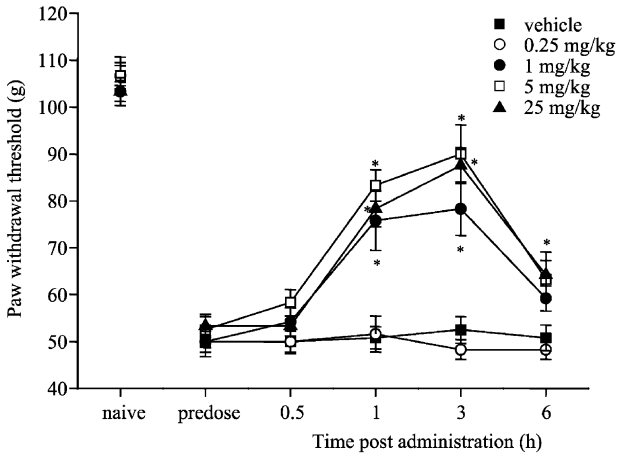

Fig. 1. The effect of SB203580 on complete Freund's adjuvant-induced mechanical hyperalgesia. Paw withdrawal thresholds were measured prior to complete Freund's adjuvant injection into the hindpaw (naive), and then 24 h later prior to (predose) and up to 6 h following oral administration. Each point represents data from six animals per group. $^{*}P < 0.05$ compared to vehicle treatment by analysis of variance followed by Tukey's honest squared difference test.

effect was rapid in onset with significant activity observed within 1 h of dosing, and a maximal reversal of 69 ± 11% was achieved at 5 mg/kg, 3 h following administration. Intrathecal administration of up to 100 μg SB203580 produced no reduction in hyperalgesia, suggesting a peripheral locus of action (data not shown).

3.2. Cytokine levels in hindpaw tissue

There was a time-dependent increase in levels of interleukin-1α, interleukin-1β and tumour necrosis factor-α in ipsilateral hindpaw tissue following complete Freund's adjuvant injection with a peak at 9 h after complete Freund's adjuvant treatment (Fig. 2A–C). Interleukin-1β levels remained significantly elevated after 24 h in the ipsilateral paw compared to the contralateral paw or paws from untreated animals. Interleukin-1β levels in the contralateral paw were not different from control (Fig. 2C). By contrast, at 24 h, levels of interleukin-1α (Fig. 2A) and tumour necrosis factor-α (Fig. 2B) were not significantly different in ipsilateral or contralateral paws compared with control.

Interleukin-1β levels were then measured in hindpaw tissue from animals treated with SB203580 (25 mg/kg, p.o.) or vehicle 24 h following complete Freund's adjuvant treatment, when hyperalgesia was established and interleukin-1β levels were significantly raised in ipsilateral paws. From Fig. 2D, it can be seen that interleukin-1β levels were again significantly increased in ipsilateral paws compared to contralateral paws or those from untreated animals. There was a slight but not significant increase in interleukin-1β levels in inflamed paws 30 min after drug or vehicle treatment, and elevated levels of interleukin-1β persisted for at least 6 h after drug or vehicle administration. Levels of interleukin-1β in inflamed paws from

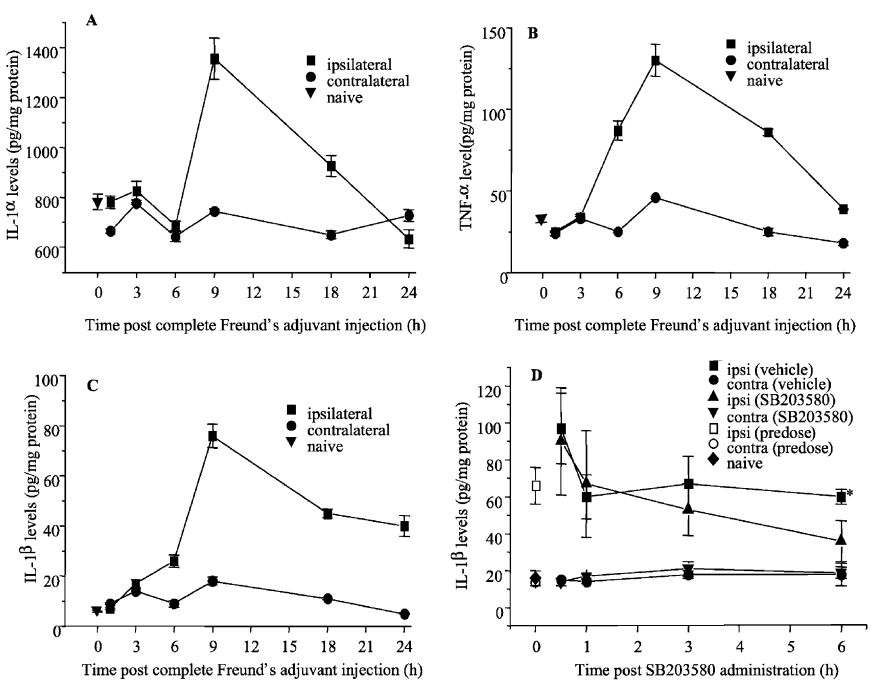

Fig. 2. Cytokine levels in inflamed hindpaw tissue. Interleukin-1α (A), tumour necrosis factor-α (B) and interleukin-1β (C) levels were measured in tissue from naive animals, and in ipsilateral and contralateral paw tissue up to 24 h following intraplantar injection of complete Freund's adjuvant. (D) The effect of SB203580 on interleukin-1β levels in the hindpaw. Levels were measured in naive paws and in ipsilateral (ipsi) and contralateral (contra) paws 24 h following complete Freund's adjuvant injection prior to (time 0) and up to 6 h following SB203580 (25 mg/kg, p.o.) or vehicle administration. Each point represents the mean of three samples taken from separate animals, each measured in triplicate. $^{*}P < 0.05$ compared to contralateral paw levels.

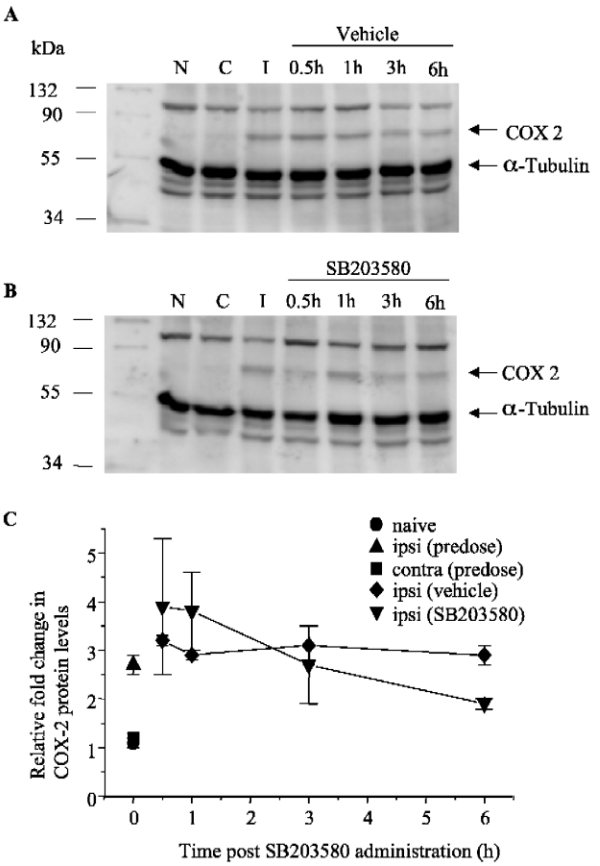

Fig. 3. Cyclo-oxygenase 2 (COX 2) levels in inflamed hindpaw tissue. Panels A and B show typical immunoblots showing cyclo-oxygenase 2 protein levels in tissue from naive (N) animals and in contralateral (C) and ipsilateral (I) paws, 24 h following complete Freund's adjuvant injection into the ipsilateral paw. Panel C shows combined data from three animals. Cyclo-oxygenase 2 levels in naive paws, and ipsilateral (ipsi) and contralateral (contra) paws prior to (predose) and following administration of SB203580 or vehicle are expressed relative to those seen in naive animals.

animals treated with SB203580 remained elevated compared to the contralateral paw, but were not significantly different from levels in inflamed paws from vehicle-treated animals at 30 min and 3 h following treatment. However, 6 h following administration of SB203580, levels of interleukin-1β were significantly reduced in the inflamed paw compared to the vehicle-treated group, and were not different from those in contralateral paws. At this time point, interleukin-1β levels in ipsilateral paws from vehicle-treated animals remained significantly different from the contralateral paws.

3.3. Cyclo-oxygenase 2 levels in hindpaw tissue

The effect of orally administered SB203580 (25 mg/kg) on cyclo-oxygenase 2 protein levels was examined by immunoblotting. Twenty-four hours following complete Freund's adjuvant treatment, the levels of cyclo-oxygenase 2 protein were significantly elevated in inflamed paws compared with non-inflamed or naive paws (Fig. 3). These elevated levels were maintained for at least 6 h and were not affected by treatment of animals with vehicle. Levels of cyclo-oxygenase 2 in SB203580-treated paws were not significantly different from those in vehicle-treated paws, although there was some reduction 6 h after administration (Fig. 3C).

3.4. Induction of bradykinin B_1 receptor-mediated hyperalgesia

Injection of interleukin-1β (1–100 ng) into the hindpaw produced a dose-related hyperalgesia that was maximal within 1 h and lasted for at least 6 h, with no change in paw withdrawal thresholds in the contralateral paw (data not shown). From this experiment, a dose of 10 ng was chosen to examine the induction of bradykinin B_1 receptor-mediated responses. Thus, 1 h following intraplantar

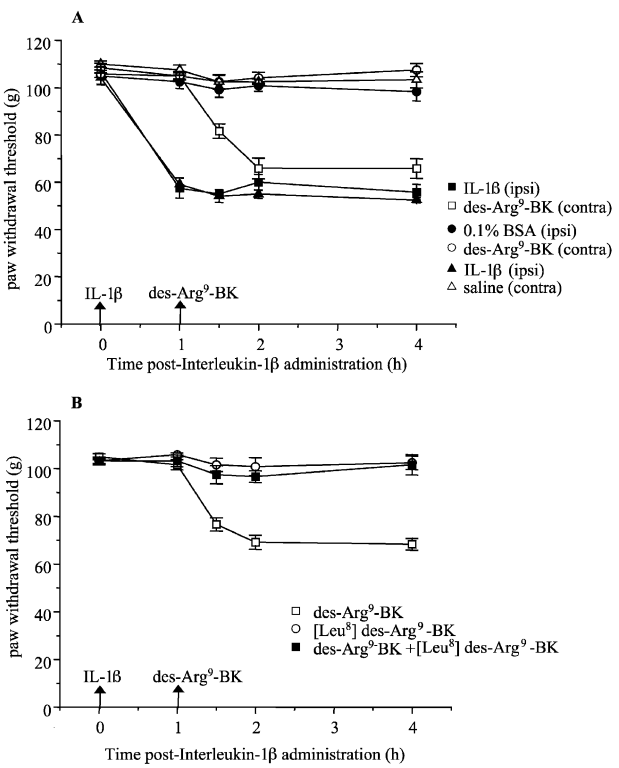

Fig. 4. Bradykinin B_1 receptor-mediated hyperalgesia. Panel A shows induction of hyperalgesia by interleukin-1β (IL-1β). Paw withdrawal thresholds were measured in both hindpaws prior to and then 1 h following administration of interleukin-1β (10 ng) or vehicle [0.1% bovine serum albumin (BSA)] into the ipsilateral paw. The bradykinin B_1 receptor agonist des-Arg^9-bradykinin (des-Arg^9-BK) (100 pmol) or vehicle (saline) was then administered into the contralateral paw and paw withdrawal thresholds measured up to 3 h later. Panel B shows antagonism of des-Arg^9-bradykinin-mediated hyperalgesia. The graph shows contralateral paw withdrawal thresholds following injection of interleukin-1β (10 ng) into the ipsilateral paw and then administration of des-Arg^9-bradykinin (100 pmol) or co-administration of des-Arg^9-bradykinin and the bradykinin B_1 receptor antagonist [Leu^8] des-Arg^9-bradykinin ([Leu^8] des-Arg^9-BK) (500 pmol) into the contralateral paw.

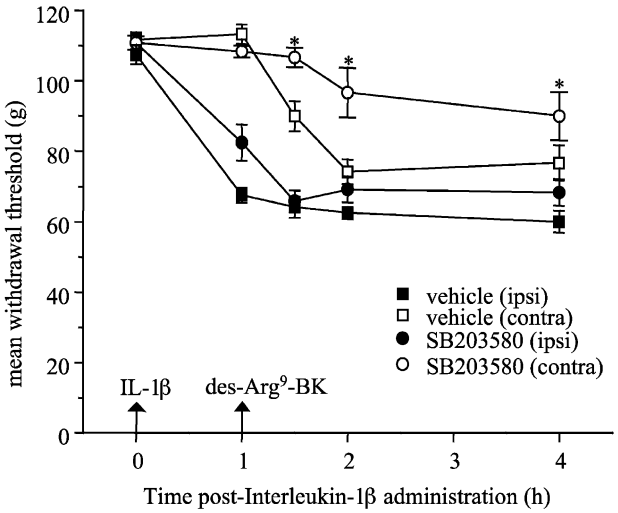

Fig. 5. The effect of SB203580 on induction of bradykinin B_1 receptor-mediated hyperalgesia. SB203580 (25 mg/kg, p.o.) or vehicle was administered to animals 1 h prior to interleukin-1β (IL-1β) (10 ng) injection into the ipsilateral hindpaw of all animals. Paw withdrawal thresholds were measured in both hindpaws prior to interleukin-1β administration, and then 1 h later prior to and up to 3 h following administration of des-Arg^9-bradykinin (des-Arg^9-BK) (100 pmol) into the contralateral paw. $^*P < 0.05$ compared to vehicle contralateral readings.

injection of 10 ng interleukin-1β, mechanical hyperalgesia was produced in the ipsilateral but not the contralateral paw (Fig. 4A). At this time, injection of the bradykinin B_1 receptor agonist des-Arg^9-bradykinin (100 pmol) into the contralateral paw elicited mechanical hyperalgesia that was evident within 30 min and lasted for at least 3 h (Fig. 4A). This des-Arg^9-bradykinin induced hyperalgesia was not seen following injection of the interleukin-1β vehicle (phosphate-buffered saline/bovine serum albumin) into the ipsilateral paw or the des-Arg^9-bradykinin vehicle (saline) into the contralateral paw (Fig. 4A). The hyperalgesia elicited by des-Arg^9-bradykinin was prevented by co-administration of the bradykinin B_1 receptor antagonist [Leu^8] des-Arg^9-bradykinin, confirming the induction of bradykinin B_1 receptor-mediated hyperalgesia by interleukin-1β (Fig. 4B).

The effect of SB203580 on the induction by interleukin-1β of bradykinin B_1 receptor-mediated hyperalgesia was then examined. In this experiment, two groups of animals received interleukin-1β injection into the ipsilateral paw and 1 h later, des-Arg^9-bradykinin into the contralateral paw. Oral administration of SB203580 1 h prior to interleukin-1β prevented the induction of the des-Arg^9-bradykinin-mediated hyperalgesia while this was still present in vehicle-treated animals (Fig. 5). A smaller but significant delay in the onset of the interleukin-1β induced hyperalgesia in the ipsilateral paw compared to vehicle was also observed. While these data suggest that the p38 stress-activated protein kinase pathway is involved in the induction of bradykinin B_1 receptor by interleukin-1β,

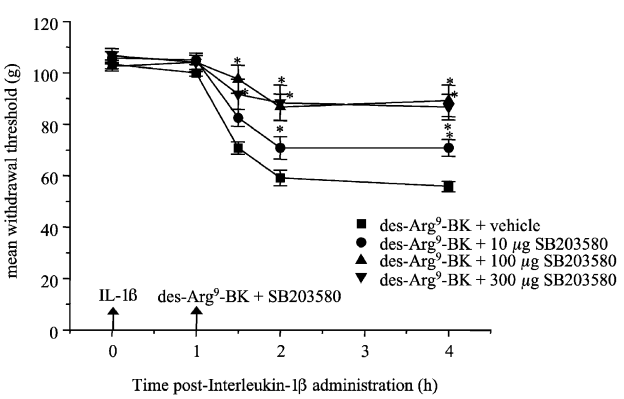

Fig. 6. The effect of SB203580 on bradykinin B_1 receptor-mediated mechanical hyperalgesia. The graph shows paw withdrawal thresholds in the contralateral paw prior to and then 1 h following administration of interleukin-1β (IL-1β) into the ipsilateral paw. Withdrawal thresholds were the measured again up to 3 h following co-administration of the bradykinin B_1 receptor agonist des-Arg^9-bradykinin (des-Arg^9-BK) (100 pmol), and SB203580 or vehicle into the contralateral paw. $^*P < 0.05$ compared to des-Arg^9-bradykinin and vehicle.

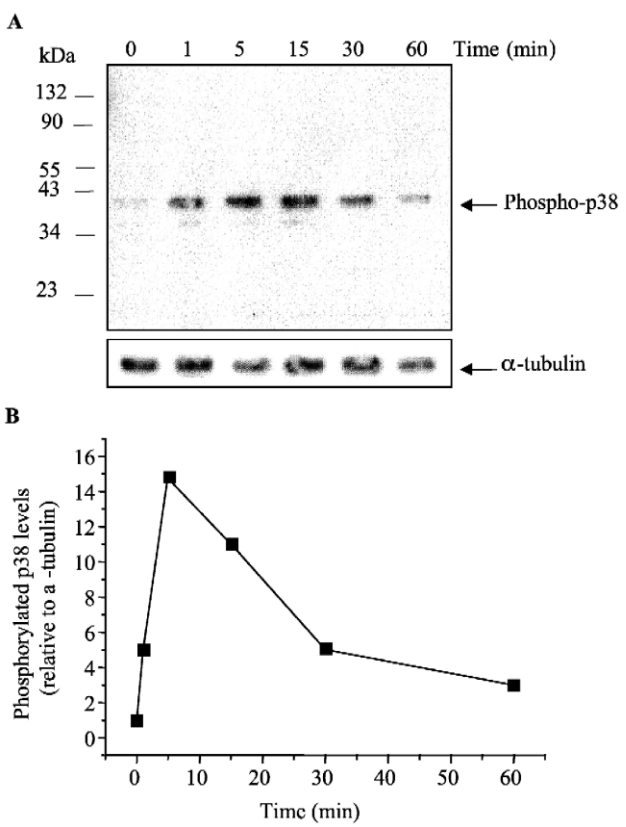

Fig. 7. Bradykinin B_1 receptor-mediated phosphorylation of p38 stress activated protein kinase. (A) Typical immunoblot showing levels of phospho-p38 stress-activated protein kinase in human embryonic kidney 293 cells, expressing the human bradykinin B_1 receptor following incubation with the bradykinin B_1 receptor agonist desArg^{10}-kallidin for 1–60 min. The immunoblot was first probed with antibodies to phospho-p38 stress-activated protein kinase, and then reprobed for α-tubulin to normalise for protein loading. (B) Levels of phospho-p38 stress-activated protein kinase normalised to α-tubulin protein in the same samples, following stimulation with desArg^{10}-kallidin.

they could conceivably also indicate that bradykinin B_1 receptors utilise the p38 stress-activated protein kinase signalling pathway. The latter possibility is supported by data shown in Fig. 6, which shows that co-administration of SB203580 with des-Arg^9-bradykinin into the contralateral paw produces a dose-dependent reduction of the bradykinin B_1 receptor-mediated hyperalgesia.

3.5. Bradykinin B_1 receptor-mediated p38 stress-activated protein kinase phosphorylation

Stimulation of human bradykinin B_1 receptor constitutively expressed in human embryonic kidney 293 cells with 1 μM des-Arg^{10}-kallidin, the preferred human bradykinin B_1 receptor agonist, resulted in a rapid phosphorylation of p38 stress-activated protein kinase endogenously expressed in this cell line (Fig. 7). A 15-fold upregulation of phosphorylation of p38 stress-activated protein kinase was observed after 5 min of stimulation with desArg^{10}-kallidin relative to unstimulated cells. These data suggest that bradykinin B_1 receptor can signal through p38 stress-activated protein kinase in this cell line.

4. Discussion

p38 stress-associated protein kinase is a key component of inflammatory processes stimulated by inflammatory insults. This kinase exists as a number of isoforms and mediates signals from pro-inflammatory cytokines to the transcription factor, activating protein-1, thus triggering events leading to inflammation. SB203580 is a selective inhibitor of p38 stress-activated protein kinase that competes with ATP for binding to the p38α and p38β isoforms and prevents phosphorylation of downstream effectors. SB203580 is inactive towards p38γ and p38δ isoforms and has no effect on jun N-terminal kinases (Lee et al., 1994; Lee and Young, 1996). It inhibits lipopolysaccharide-induced cytokine production in vitro and in vivo and has been shown to be effective in models of arthritis, endotoxic shock and inflammatory angiogenesis (Badger et al., 1996; Jackson et al., 1998). Here, we demonstrate for the first time that SB203580 reverses established mechanical hyperalgesia in a model of inflammatory pain following oral administration. It was highly efficacious, and its effect was rapid in onset and longlasting. It is notable that despite the fact that the p38 stress-activated protein kinase pathway is only one of three, besides jun N-terminal kinases and nuclear factor κ-B, activated by lipopolysaccharide, cytokines, stress etc., its inhibition has such a significant effect in reversing hyperalgesia.

As mentioned earlier, the inflammatory cytokines interleukin-1β and tumour necrosis factor-α have been shown to produce hyperalgesia in a number of models. Previous studies have reported elevated levels of both cytokines in paw tissue following complete Freund's adjuvant injection (Woolf et al., 1997). In the present study, we confirm these findings, although with some differences in the time course. Thus, we found that interleukin-1β levels were raised in complete Freund's adjuvant-treated paws for at least 24 h, while tumour necrosis factor-α levels reached a peak at 9 h and then declined. These observations differ from those of Woolf et al. (1997), who saw peak tumour necrosis factor-α levels at 24 h, and the levels remained elevated for 5 days after complete Freund's adjuvant injection. The main reason for this discrepancy is likely to be due to the lower dose of complete Freund's adjuvant (25%) used in our study, which probably caused a less severe inflammatory response than that observed by Woolf et al. (1997). The inhibition of cytokine signalling by SB203580 is a likely mechanism for its observed antihyperalgesic activity. There are likely to be several mechanisms by which cytokines contribute to hyperalgesia, including a direct activation or sensitisation of primary afferent neurones (Sorkin et al., 1997) and induction of other proteins and mediators. This may include further cytokine production, and these may then in turn activate p38 stress-activated protein kinase in a positive feedback loop (Lee et al., 1994; Lee and Young, 1996). A suppression of this feedback loop could thereby contribute to the antihyperalgesic activity of SB203580. However, we found no correlation of inhibition of interleukin-1β levels and reversal of hyperalgesia by the inhibitor. Thus, interleukin-1β levels were reduced only at 6 h following administration of SB203580, while by this time point, hyperalgesia had returned to predose levels.

A possible key protein induced by cytokines is cyclo-oxygenase 2. It is induced in tissues and cells by lipopolysaccharide, and interleukin-1β and has been proposed to play a key role in inflammatory hyperalgesia through the production of prostaglandins, which ultimately sensitise sensory neurones (Chan et al., 1995). In the present study, we showed that cyclo-oxygenase 2 protein was absent in naive paw tissue, but was increased more than 3-fold in inflamed tissue. SB203580 did not, however, significantly reduce cyclo-oxygenase 2 levels in inflamed tissue. Although we did not measure prostanoid levels, there are no published reports of a direct inhibition of cyclo-oxygenase 2 by SB203580, and it is therefore unlikely that at the doses used here, a direct inhibition of enzyme activity could account for the reversal of hyperalgesia seen in the inflammatory models.

The bradykinin B_1 receptor, like cyclo-oxygenase 2, is not constitutively expressed, but is induced during conditions of inflammation. Its expression is induced in isolated tissues and cells by interleukin-1β and tumour necrosis factor-α treatment, and in vivo by lipopolysaccharide administration (see Marceau et al., 1998 for review). Several studies have demonstrated that bradykinin B_1 receptors play a role in inflammatory hyperalgesia. Thus, bradykinin B_1 receptor antagonists inhibit thermal hyperalgesia fol-

lowing ultraviolet irradiation of the paw and mechanical hyperalgesia in the knee joint elicited by complete Freund's adjuvant, interleukin-1β or capsaicin (Perkins et al., 1993; Perkins and Kelly, 1993; Davis and Perkins, 1996). Furthermore, while bradykinin B_1 receptor agonists do not normally affect nociceptive thresholds in animal models, they have been shown to evoke hyperalgesia in the knee joint following treatment with interleukin-1β (Davis and Perkins, 1993, 1994; Perkins and Kelly, 1994). In the present study, we show that interleukin-1β, as expected, produces mechanical hyperalgesia in the hindpaw. In addition, following interleukin-1β treatment, the bradykinin B_1 receptor agonist des-Arg^9-bradykinin produces mechanical hyperalgesia in the contralateral paw, suggesting a systemic induction of bradykinin B_1 receptor by interleukin-1β. This apparent induction of bradykinin B_1 receptors was prevented by SB203580 administered prior to the injection of interleukin-1β into the paw. However, it also inhibited the acute hyperalgesia elicited by des-Arg^9-bradykinin injection into the contralateral paw, indicating a role for p38 stress-activated protein kinase in the bradykinin B_1 receptor-mediated hyperalgesia. This mechanism is independently supported by in vitro data, showing that activation of human bradykinin B_1 receptors expressed in human embryonic kidney 293 cells with desArg^{10}-kallidin rapidly resulted in phosphorylation of p38 stress-activated protein kinase expressed in this cell line. These findings suggest that p38 stress-activated protein kinase is involved in the bradykinin B_1 receptor induction by interleukin-1β, and also that the bradykinin B_1 receptor itself signals through this pathway. Therefore, SB203580 could conceivably be acting at both these points to elicit its anti-hyperalgesic effect. Similar findings have been reported by Haddad et al. (2000), who showed that SB203580 inhibited the induction of bradykinin B_1 receptor mRNA in fibroblasts by interleukin-1β or tumour necrosis factor-α. In addition, the time-dependent induction of bradykinin B_1 receptor-mediated contractions in the isolated rabbit aorta has been shown to be inhibited by SB203580 (Larrivée et al., 1998). While in this study, the inhibitor did not acutely affect contractions elicited by des-Arg^9-bradykinin, other reports support our findings that bradykinin B_1 receptor may also signal via the p38 stress-activated protein kinase pathway. For example, Wilk-Blaszczak et al. (1998) have shown that bradykinin inhibits calcium currents in neuroblastoma cells via activation of the p38-2 stress-activated protein kinase isoform, and bradykinin B_1 and B_2 receptors expressed in human embryonic kidney 293 cells have been shown to activate the activating protein-1 transcription factor via the mitogen-activated protein kinase pathway (Naraba et al., 1998). Given that activating protein-1 is also activated by p38 stress-activated protein kinase, it is possible that bradykinin B_1 receptors signal via this pathway.

In conclusion, we have demonstrated that the p38 stress-activated protein kinase inhibitor, SB203580, reverses inflammatory mechanical hyperalgesia, and that this effect is at least partly due to both the induction of bradykinin B_1 receptor function, as well as an inhibition of the bradykinin B_1 receptor signal transduction pathway.

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