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RESEARCH PAPER

# Fructose-1,6-bisphosphate reduces inflammatory pain-like behaviour in mice: role of adenosine acting on $A_1$ receptors

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**Background and purpose:** D-Fructose-1,6-bisphosphate (FBP) is an intermediate in the glycolytic pathway, exerting pharmacological actions on inflammation by inhibiting cytokine production or interfering with adenosine production. Here, the possible antinociceptive effect of FBP and its mechanism of action in the carrageenin paw inflammation model in mice were addressed, focusing on the two mechanisms described above.
**Experimental approach:** Mechanical hyperalgesia (decrease in the nociceptive threshold) was evaluated by the electronic pressure-metre test; cytokine levels were measured by ELISA and adenosine was determined by high performance liquid chromatography.
**Key results:** Pretreatment of mice with FBP reduced hyperalgesia induced by intraplantar injection of carrageenin (up to 54%), tumour necrosis factor α (40%), interleukin-1 β (46%), CXCL1 (33%), prostaglandin $E_2$ (41%) or dopamine (55%). However, FBP treatment did not alter carrageenin-induced cytokine (tumour necrosis factor α and interleukin-1 β) or chemokine (CXCL1) production. On the other hand, the antinociceptive effect of FBP was prevented by systemic and intraplantar treatment with an adenosine $A_1$ receptor antagonist (8-cyclopentyl-1,3-dipropylxanthine), suggesting that the FBP effect is mediated by peripheral adenosine acting on $A_1$ receptors. Giving FBP to mice increased adenosine levels in plasma, and adenosine treatment of paw inflammation presented a similar antinociceptive mechanism to that of FBP.
**Conclusions and implications:** In addition to anti-inflammatory action, FBP also presents an antinociceptive effect upon inflammatory hyperalgesia. Its mechanism of action seems dependent on adenosine production but not on modulation of hyperalgesic cytokine/chemokine production. In turn, adenosine acts peripherally on its $A_1$ receptor inhibiting hyperalgesia. FBP may have possible therapeutic applications in reducing inflammatory pain.
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**Keywords:** D-Fructose-1,6-bisphosphate; pain; cytokine; adenosine; DPCPX

**Abbreviations:** DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; FBP, D-Fructose-1,6-bisphosphate; HPLC, high-performance liquid chromatography; KC, CXCL1, keratinocyte-derived chemokine; IL-1β, interleukin-1 β; $PGE_2$, prostaglandin $E_2$; TNFα, tumour necrosis factor α

## Introduction

The glycolytic intermediate, D-Fructose-1,6-bisphosphate (FBP), is a high-energy physiological meatabolite that exhibits pharmacological activity. For instance, FBP has a protective effect on organ and tissue damage observed after ischaemia/reperfusion of liver (Mihas *et al.*, 2003), ameliorates hepatic disfunction during galactosamine-induced experimental

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hepatitis (De Oliveira *et al.*, 1992), and its addition to storage solution increases the preservation of the liver for transplantation (Moresco *et al.*, 2004). Furthermore, FBP also reduced inflammatory parameters such as carrageenin-induced paw oedema (Planas *et al.*, 1993) and pleurisy (Alves-Filho *et al.*, 2004).

The pharmacological anti-inflammatory activity of FBP seems to be related to inhibition of the production of inflammatory molecules including intracellular reactive oxygen species, prostaglandin $E_2$ ($PGE_2$, Ahn *et al.*, 2002), cytokines (e.g. tumour necrosis factor α, TNFα; Hirokawa *et al.*, 2002; Markov *et al.*, 2002; Tamaki *et al.*, 2002; Bordignon Nunes *et al.*, 2003; Cohly *et al.*, 2004; Cuesta *et al.*, 2006; Lopes *et al.*, 2006) and cycloxygenase-2 expression (Ahn *et al.*, 2002). In addition, infusion of FBP decreases xanthine accumulation during the ischaemic period in ischaemia/reperfusion models, thus inhibiting neutrophil recruitment and subsequent neutrophil free-radical generation during reperfusion (Sola *et al.*, 2003). There is also evidence suggesting that adenosine mediates these anti-inflammatory actions of FBP, as the effects of FBP in the ischaemia/reperfusion model were reversed by treatment with adenosine deaminase, which is the enzyme that converts adenosine to an inactive metabolite (Sola *et al.*, 2003). In agreement, adenosine and FBP presented similar inhibitory action profiles in ischaemia/reperfusion-induced leukocyte adherence and microvascular dysfunction in skeletal muscle (Akimitsu *et al.*, 1995). Such evidence strongly suggests a therapeutic use of FBP in the treatment of inflammatory conditions.

One of the most important symptoms of the inflammatory process is the increase in pain sensitivity, which is referred to as hyperalgesia (Verri *et al.*, 2006a; 2007a; Cunha *et al.*, 2007). This inflammatory phenomenon is mediated by a large number of mediators including cytokines (Ferreira *et al.*, 1988; Cunha *et al.*, 1991; 1992; Verri *et al.*, 2004; 2005; 2006b; 2007b; 2008a) and also by inflammatory cells such as neutrophils (Levine *et al.*, 1984; Verri *et al.*, 2007c; 2009; Cunha *et al.*, 2008a; Guerrero *et al.*, 2008; Ting *et al.*, 2008). These mediators and cells are ultimately responsible for the release of the directly acting hyperalgesic mediators that act on receptors present in the membrane of primary nociceptive neurons leading to their sensitization (Verri *et al.*, 2006b; Cunha *et al.*, 2007). Despite all the evidence that FBP presents anti-inflammatory properties, there are no data showing the possible antinociceptive effect of FBP. Therefore, in the present study, the possible antinociceptive effect of FBP and its mechanism of action in the carrageenin model of paw inflammation in mice were addressed, focusing mainly on the modulation of production of adenosine and hyperalgesic cytokines.

## Methods

*Animals*

All animal care and experimental procedures were in accordance with the National Institute of Health guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Medicine of Ribeirao Preto (University of Sao Paulo). Adult male Swiss mice (22–28 g) were obtained from the University of Sao Paulo, campus of Ribeirao Preto and housed in a temperature-controlled room with access to water and food *ad libitum* until use. All experiments were designed with a double blind format.

*Mechanical hyperalgesia evaluation*

Mechanical hyperalgesia was tested in mice as previously reported (Cunha *et al.*, 2004). 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 hindpaw flexion reflex with a hand-held force transducer (electronic anaesthesiometer; IITC Life Science, Woodland Hills, CA, USA) adapted with a 0.5 $mm^2$ polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw 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 averaging of three measurements. The animals were tested before and after treatments. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements 3 h after stimulus. Withdrawal threshold was 9.2 ± 0.5 g (mean ± SEM; $n = 30$) before injection of the hyperalgesic agents (e.g. cytokines or carrageenin).

*Experimental protocol*

Mechanical hyperalgesia was measured in the mice before and after the intraplantar injection of one of the following substances: carrageenin (100 μg), TNFα (100 pg), the chemokine, CXCL1 (40 ng), interleukin (IL)-1β (1 ng), dopamine (10 μg), $PGE_2$ (30 μg) or saline (the vehicle of the inflammatory mediators used, 25 μL). The mechanical hyperalgesia was determined 3 h after the intraplantar injection of stimulus, except for Figure 1D in which hyperalgesia was evaluated 1–5 h after carrageenin injection. FBP was given via i.p., p.o. or s.c. routes 15 min before (100–1000 $mg \cdot kg^{-1}$) or 1 h after (300 $mg \cdot kg^{-1}$ via i.p. route) the carrageenin intraplantar stimulus (Figure 1), and the vehicle control was saline. In the other experiments, the FBP dose was 300 $mg \cdot kg^{-1}$ via i.p. route 15 min before stimulus. Treatment with DPCPX (8-cyclopentyl-1,3-dipropylxanthine; an adenosine $A_1$ receptor antagonist) via s.c. (3–30 $mg \cdot kg^{-1}$) or intraplantar (0.3–3.0 μg per paw) routes was performed 15 min before further treatment with FBP (300 $mg \cdot kg^{-1}$) or adenosine (100 $mg \cdot kg^{-1}$), then after additional 15 min mice received the stimulus injection (intraplantar $PGE_2$, dopamine or carrageenin). A scheme of treatment is presented within Figures 4 and 6 in which DPCPX was used. Cytokine (TNFα and IL-1β) and chemokine (CXCL1) levels were determined 3 h after intraplantar injection of carrageenin (100 μg). Adenosine levels were determined by high-performance liquid chromatography (HPLC) 1, 2 and 3 h after FBP or adenosine treatment. All the drugs were dissolved in saline except DPCPX, which was dissolved in Tween 80 0.5% in saline. The intraplantar injections were made in a volume of 25 μL per paw, while i.p. and s.c. injections were in a volume of 200 μL per 20 g of body weight and p.o. injection in a volume of 100 μL per 20 g of body weight. Doses of stimuli and time for evaluation were previously determined in our laboratory (Cunha *et al.*, 2004; 2005; 2008b; Verri *et al.*, 2008b).

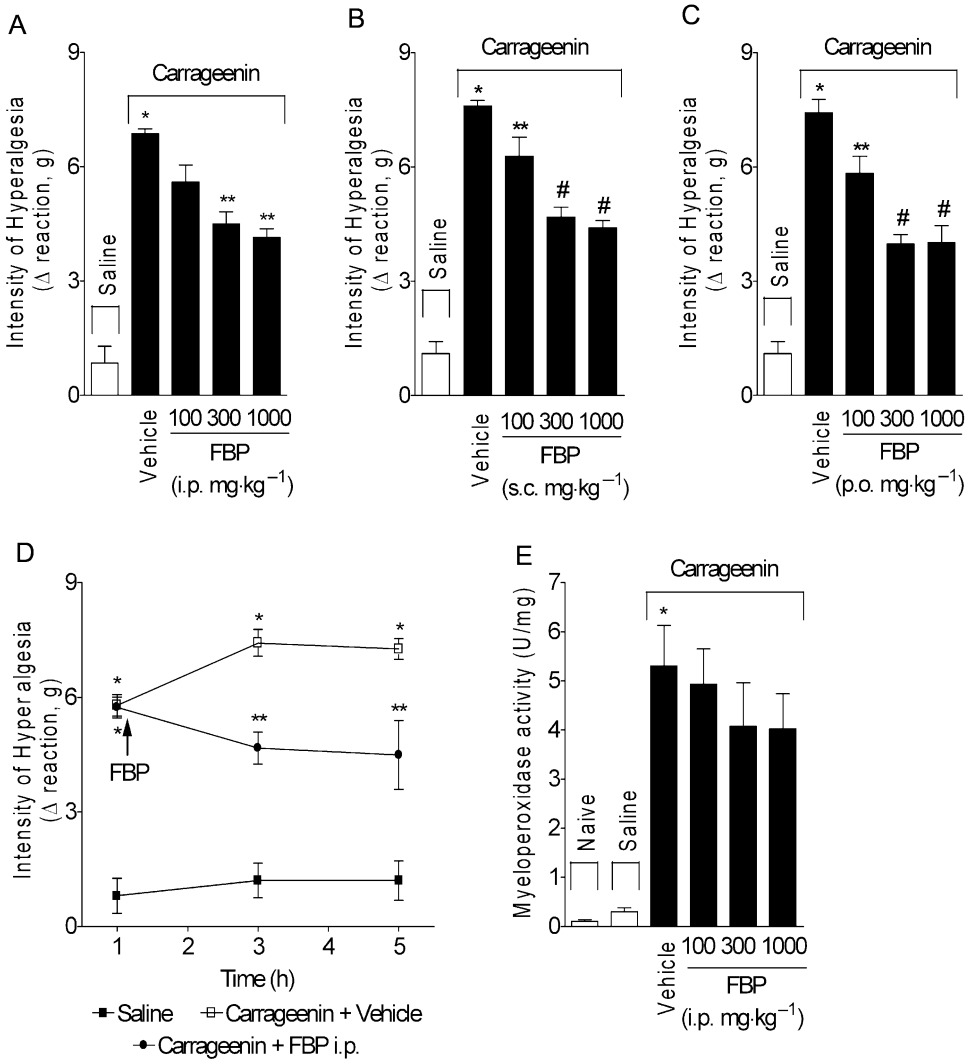


**Figure 1** D-Fructose-1,6-bisphosphate (FBP) inhibits carrageenin-induced mechanical hyperalgesia independently of the route of treatment. (A–C) Mice were treated with FBP (100–1000 mg·kg$^{-1}$, 15 min) or vehicle (saline) via i.p. (A), s.c. (B) or p.o. (C) before the intraplantar carrageenin (100 μg per paw) stimulus or saline. (D) Mice were treated with FBP (300 mg·kg$^{-1}$, i.p.) or vehicle (saline) 1 h after carrageenin or saline. The intensity of hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-metre test in (A–C) or 1–5 h in (D). (E) Mice were treated with FBP (100–1000 mg·kg$^{-1}$, i.p., 15 min) or vehicle (saline) before the intraplantar carrageenin or saline. The myeloperoxidase activity was measured in samples of s.c. plantar tissue collected 3 h after carrageenin injection. $n = 5$ mice per group per experiment, representative of two separate experiments. *$P < 0.05$ compared with the saline group, **$P < 0.05$ compared with the vehicle group and $^{\#}P < 0.05$ compared with the FBP 100 mg·kg$^{-1}$ groups (one-way ANOVA followed by Tukey's *t*-test).

*Measurement of motor performance*

In order to exclude possible non-specific, muscle relaxant or sedative effects of FBP, motor performance of mice was evaluated by the rota-rod test (Kuribara *et al.*, 1977). The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by discs of 25 cm in diameter (Ugo Basile, Model 7600, Comerio, VA, Italy). The bar rotated at a constant speed of 22 revolutions per minute. The animals were selected 24 h previously by eliminating those mice that could not remain on the bar for two consecutive periods of 180 s. Animals were treated with vehicle (saline) or FBP (300 mg·kg$^{-1}$, i.p.) for 3 h and 15 min (this time is equivalent to the period of mechanical hyperalgesia evaluation) before testing. The cut-off time used was 180 s.

*Cytokine measurements*

At 3 h after the injection of inflammatory stimuli, mice were terminally anaesthetized, and the skin tissues were removed from the injected and control paws (saline and naïve). The samples were homogenized in 500 μL of the following buffer containing protease inhibitors: NaCl 0.4 M, Tween 20 0.05%, bovine albumin 0.5%, phenyl methyl sulphonyl fluoride 0.1 mM, benzethonium chloride 0.1 mM, EDTA 10 mM, aprotinin 20 KI·mL$^{-1}$ (0.01 mg·mL$^{-1}$) diluted in phosphate buffer saline pH 7.4. The TNFα, CXCL1 and IL-1β levels were determined as described previously (Valerio *et al.*, 2007) by enzyme-linked immunosorbent assay (ELISA), and the results were expressed as pg cytokine·(g paw skin tissue)$^{-1}$. As a control, the levels of these cytokines were determined in naive mice and animals injected with saline.

*Adenosine quantification*
A sensitive, reproducible and quantitative HPLC-UV detection method for adenosine was developed. The advantages of HPLC for this analysis are its versatility and simplicity of sample preparation, as well as a broad linearity in detectors, making HPLC the method of choice for this purpose. The analysis was performed on HPLC system consisting of a Shimadzu Model (Kyoto, Japan) LC 10 AD pump, a Shimadzu Model SPD-10A ultraviolet detector and a chromatopac C-R6A integrator (Shimadzu). Chromatographic separation was achieved at room temperature on a LiChrospher 100 RP-18 column [125× 4 mm, 5 μm particle size (Merck, Damstadt, Germany)]. The mobile phase consisted of 0.5% of acetonitrile, 4.5% of methanol and 95% of sodium acetate buffer 0.25 M, pH 6.5. HPLC-grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. Flow rate was 1.0 mL $min^{-1}$, and the ultraviolet detector was set at 254 nm.

An adenosine standard curve (0.2–6 $\mu g \cdot mL^{-1}$) was constructed in plasma samples of saline (vehicle)-treated animals. The acceptance criterion for a calibration curve was a correlation coefficient ($R^2$) of at least 0.99. To correct for possible losses of adenosine during sample preparation, 2 μg of theophylline was used as internal standard. For this purpose 25 μL of 0.08 $mg \cdot mL^{-1}$ theophylline solution was added to the plasma as described below. Aqueous adenosine and theophylline standard was used to confirm the retention time and the required run-time. The stock solution of adenosine at 4 $mg \cdot mL^{-1}$ was prepared in methanol and stored at –20°C, and further diluted in plasma to yield the indicated concentrations. The stock solution of theophylline (40 $\mu g \cdot mL^{-1}$) was also prepared in methanol and stored at –20°C until further dilution in plasma.

Mice were treated with FBP (300 $mg \cdot kg^{-1}$, i.p.) or vehicle (saline), and after 2 or 3 h, mice were anaesthetized and blood samples collected by cardiac puncture with heparin were kept in ice. The time points were after FBP treatment. Blood samples were centrifuged for 15 min at 1600× *g*; 500 μL of the resulting plasma was separated, and theophylline was added as a control for retention time in the subsequent HPLC analysis. One group of plasma samples from mice receiving vehicle only had adenosine added to them as a positive controls for the detection of adenosine in the HPLC analysis. Then 1 mL of acetonitrile was added to each tube with 500 μL of plasma sample, followed by 2 min vortex agitation to precipitate plasma proteins and centrifugation. The supernatant was separated and dried under air flow. The resulting pellet was resuspended in 150 μL of mobile phase (0.5% of acetonitrile, 4.5% of methanol and 95% of sodium acetate buffer 0.25 M, pH 6.5) plus 100 μL of hexane. Again, the sample was agitated for 30 s and centrifuged for 5 min at 1600× *g*. Twenty microlitres of the water phase (lower phase) were used for chromatographic analysis. Adenosine and theophylline standards were used to confirm the retention time and the required run-time, and adenosine was identified by the retention time, compared with that of standard theophylline and adenosine. A group of mice were treated with adenosine (100 $mg \cdot kg^{-1}$), and 2 and 3 h after treatment samples were collected and processed as described for FBP-treated mice.

*Myeloperoxidase activity*
The myeloperoxidase kinetic–colorimetric assay was used to evaluate the leukocyte migration to the s.c. plantar tissue of mice hind paw (Bradley *et al.*, 1982; Casagrande *et al.*, 2006). Samples of s.c. plantar tissue were collected at 3 h after carrageenin injection in 50 mM $K_2HPO_4$ buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide and kept at –80°C until use. Samples were homogenized by using a Polytron (PT3100), centrifuged at 16 100× *g* for 4 min and the resulting supernatant assayed spectrophotometrically for myeloperoxidase activity determination at 450 nm (Spectra max), with three readings in 1 min. First, the results were reported as total number of neutrophils by comparing absorbance of the tissue supernatant with that of mice peritoneal neutrophils processed in the same way. To this end, neutrophil migration was induced in the peritoneum of mice by injecting carrageenin (500 μg per animal). A standard curve relating neutrophil numbers [90% purity, 200 000 to 781 neutrophils·$(50\ \mu L)^{-1}$] and absorbance was obtained by processing purified neutrophils, as described above, and assaying for myeloperoxidase activity. The correlation between the number of neutrophils and units of myeloperoxidase was determined by using the technique described by Bradley *et al.* (1982). The neutrophil standard curve was processed by using 0.0005% hydrogen peroxide as substrate for myeloperoxidase. A unit of myeloperoxidase activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 22°C (Bradley *et al.*, 1982)

*Statistical analysis*
Results are presented as means ± SEM for groups of five animals (for *in vivo* experiments) or four animals (for *ex vivo* experiments), and they are representative of two independent experiments. The differences between the experimental groups were compared by one-way ANOVA followed by Tukey's *t*-test. The level of significance was set at $P < 0.05$.

*Materials*
The following materials were obtained from the sources indicated. Recombinant murine TNFα and IL-1β were provided by The National Institute for Biological Standards and Control (NIBSC, South Mimms, Hertfordshire, UK). Recombinant murine CXCL1 was purchased from PeproTech Inc., (Rocky Hill, NJ, USA), carrageenin from FMC Corporation (Philadelphia, PA, USA), and adenosine, DPCPX and FBP from Sigma (St. Louis, MO, USA).

## Results

*FBP reduced carrageenin-induced mechanical hyperalgesia independently of the administration route*
Mice were treated with FBP (100, 300 or 1000 $mg \cdot kg^{-1}$) or vehicle (saline, 200 μL to each 20 g of mice) via i.p. (Figure 1A), s.c. (Figure 1B) or p.o. (Figure 1C) routes 15 min before the intraplantar injection of carrageenin (100 μg per paw) (Cunha *et al.*, 2004) or vehicle (saline, 25 μL) injection. The mechanical hyperalgesia induced by carrageenin was

dose-dependently reduced by FBP treatments via i.p., s.c. and p.o. routes, to a maximum effect of 45, 49, 54% respectively (Figure 1A–C). Compared with vehicle group, there were significant differences with the doses of 300 and 1000 mg·kg$^{-1}$, and no significant effect with the dose of 100 mg·kg$^{-1}$ (Figure 1A, i.p. treatment). There was no difference between the doses of 300 and 1000 mg·kg$^{-1}$, therefore the dose of 300 mg·kg$^{-1}$ was used in the subsequent experiments. The i.p. treatment with 300 mg·kg$^{-1}$ of FBP given 3 h and 15 min before testing did not alter the motor performance of mice ($n$ = 6). The vehicle control response in the rota-rod test was 180 s versus 180 s of FBP-treated animals respectively (data not shown). Furthermore, this dose of FBP did not alter the mechanical baseline of mice (data not shown). With regard to the s.c. (Figure 1A) and p.o. (Figure 1B) routes of FBP treatment, there was significant reduction of carrageenin-induced mechanical hyperalgesia with the dose of 100 mg·kg$^{-1}$. The doses of 300 and 1000 mg·kg$^{-1}$ had similar effects, which were significantly different compared from that of vehicle and the dose of 100 mg·kg$^{-1}$.

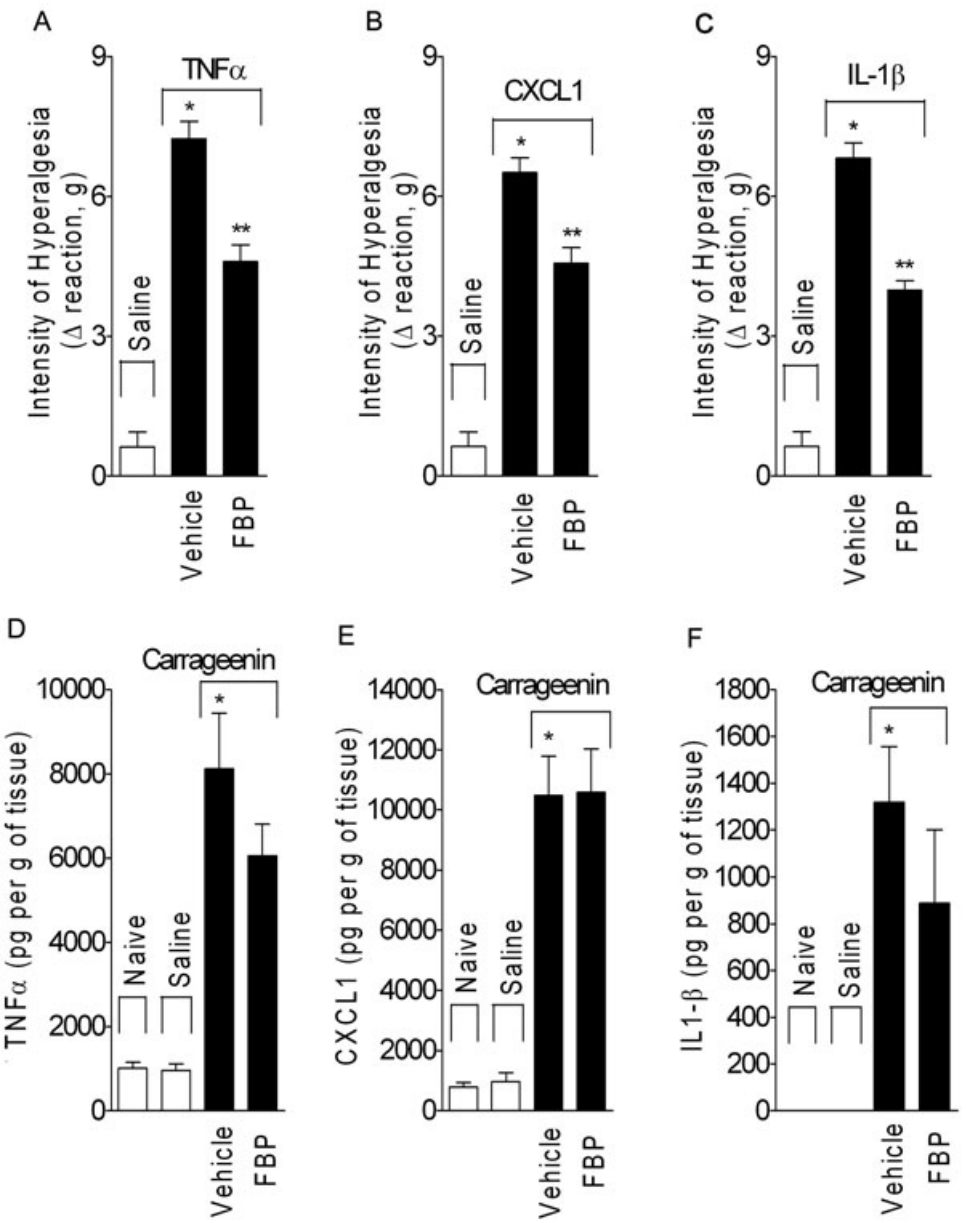


**Figure 2** D-Fructose-1,6-bisphosphate (FBP) inhibits cytokine/chemokine-induced mechanical hyperalgesia without altering carrageenin-induced cytokine/chemokine production. (A–C) Mice were treated with FBP (300 mg·kg$^{-1}$, i.p., 15 min) or vehicle (saline) before the intraplantar injection of tumour necrosis factor α (TNFα (A; 100 pg per paw), CXCL1 (B; 40 ng per paw) or interleukin-1 β (IL-1β) (C; 1 ng per paw). The intensity of hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-metre test. (D–F) Mice were treated with FBP (300 mg·kg$^{-1}$, i.p., 15 min) or vehicle (saline) before carrageenin (100 μg per paw) or saline. The samples of s.c. plantar tissue were collected 3 h after stimulus and processed for TNFα (D), CXCL1 (E) and IL-1β (F) quantification by ELISA. $n$ = 5 mice per group per experiment, representative of two separate experiments. *$P$ < 0.05 compared with the saline group and **$P$ < 0.05 compared with the vehicle group (one-way ANOVA followed by Tukey's $t$-test).

In panels A–C of Figure 1, mice were pretreated with FBP and in Figure 1D, the results of treatment with FBP *after* the carrageenin are shown. Mice received the intraplantar injection of carrageenin or saline and mechanical hyperalgesia was determined at 1 h. There were significant differences in hyperalgesia between the saline and carrageenin groups at this time. After measurement, the group of mice that received carrageenin was split into two groups ($n$ = 5 each): one was treated with saline and the other with FBP (300 mg·kg$^{-1}$, i.p.). The mechanical hyperalgesia was then re-evaluated at 3 and 5 h after carrageenin injection. As shown, this later treatment with FBP reduced the carrageenin-induced mechanical hyperalgesia at both time points (Figure 1D).

Neutrophil recruitment and activation at the inflammatory focus is a critical step in the establishment of hyperalgesia (Levine *et al*., 1984; Guerrero *et al*., 2008; Ting *et al*., 2008). In our model, i.p. treatment with 300 mg·kg$^{-1}$ of FBP (15 min before carrageenin) did not alter the carrageenin-induced neutrophil migration to the paw skin, as measured by myeloperoxidase activity, 3 h after carrageenin injection (Figure 1E).

*FBP reduced cytokines- and chemokine-induced mechanical hyperalgesia without altering carrageenin-induced cytokines and chemokine production*

The release of cytokines, such as TNFα and IL-1β, and chemokines such as CXCL1 constitutes an important event in the development of inflammatory hyperalgesia (Cunha *et al*., 2005). Therefore, the effects of FBP on the mechanical hyperalgesia induced by cytokines and chemokines were evaluated. At the antinociceptive dose determined in Figure 1A (300 mg·kg$^{-1}$, 15 min, i.p.), FBP treatment reduced the mechanical hyperalgesia induced by injection of TNFα (100 pg per paw, 40%, Figure 2A), CXCL1 (40 ng per paw, 25%, Figure 2B) and IL-1 β (1 ng per paw, 46%, Figure 2C) at 3 h after stimulus injection.

Because FBP treatment inhibits cytokine production (Tamaki *et al*., 2002; Bordignon Nunes *et al*., 2003), we tested FBP as an inhibitor of cytokine/chemokine production in our model. Our results showed that treatment of mice with an antinociceptive dose of FBP (300 mg·kg$^{-1}$, i.p.) did not alter the carrageenin-induced production of TNFα (Figure 2D), CXCL1 (Figure 2E) and IL-1β (Figure 2F) in paw tissue.

*FBP reduced mechanical hyperalgesia induced by directly acting hyperalgesic mediators: $PGE_2$ and sympathomimetic amines*

We and others have demonstrated that the hyperalgesic effect of cytokines depends on the production of two directly acting mediators, prostaglandins and sympathomimetic amines (see Verri *et al*., 2006b). While TNFα, CXCL1 and IL-1β induce hyperalgesia by induction of $PGE_2$, CXCL1 also acts through release of sympathomimetic amines (Cunha *et al*., 2005). In our present model, we found that pretreatment of mice with FBP (300 mg·kg$^{-1}$, i.p.) reduced either $PGE_2$- or dopamine-induced mechanical hyperalgesia by 41% and 55% respectively (Figure 3).

*The selective adenosine $A_1$ receptor antagonist, DPCPX, prevents the antinociceptive effects of FBP and adenosine on mechanical hyperalgesia induced by carrageenin, $PGE_2$ and dopamine*

Some pharmacological effects of FBP are mediated by adenosine (Sola *et al*., 2003), and peripheral activation of adenosine $A_1$ receptors inhibits mechanical hyperalgesia produced by $PGE_2$ (Taiwo and Levine, 1990). In our model, systemic treatment of mice with DPCPX (a selective adenosine $A_1$ receptor antagonist; 3–30 mg·kg$^{-1}$, 15 min, i.p.) prevented, in a dose-dependent manner, the antinociceptive action of FBP (300 mg·kg$^{-1}$, 15 min, i.p.) on carrageenin-induced hyperalgesia (Figure 4A). Moreover, local, intraplantar treatment with DPCPX (0.3–3 μg per paw, 15 min) prevented the FBP (300 mg·kg$^{-1}$, 15 min, i.p.) inhibition of $PGE_2$- (Figure 4B) or dopamine- (Figure 4C) induced hyperalgesia. Importantly, the preventive effect of locally administered DPCPX upon FBP antinociceptive activity in $PGE_2$ and dopamine hyperalgesia (Figure 4B,C) was observed only when DPCPX was administered to the paw receiving the inflammatory stimulus (ipsilateral), but not when DPCPX was given to the contralateral paw (white bars of Figure 4B,C).

*Systemic administration of FBP increased adenosine plasma levels*

As the antinociceptive effect of FBP was inhibited by an adenosine $A_1$ receptor antagonist (DPCPX), we measured adenosine plasma levels, after FBP. We developed a HPLC method to quantitate adenosine in blood samples and were able to show that *in vivo* treatment with FBP or adenosine resulted in increased blood levels of adenosine (Figure 5A–D). Figure 5A shows the experimental record for control blood samples from vehicle (saline)-treated mice. The endogenous adenosine (peak 1) had a retention time of approximately

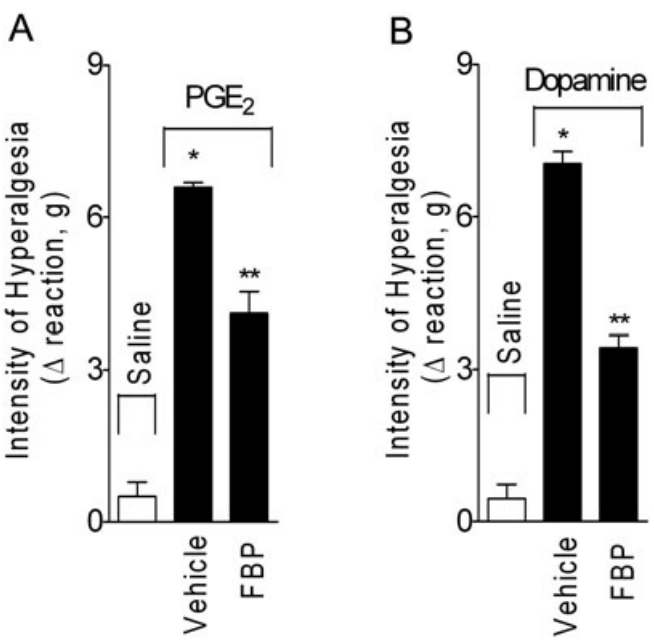


**Figure 3** D-Fructose-1,6-bisphosphate (FBP) inhibits mechanical hyperalgesia induced by final mediators: prostaglandin $E_2$ ($PGE_2$) and dopamine. Mice were treated with FBP (300 mg·kg$^{-1}$, i.p., 15 min) or vehicle (saline) before intraplantar injection of $PGE_2$ (30 ng per paw, A) or dopamine (10 μg per paw, B). The intensity of hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-metre test. $n$ = 5 mice per group per experiment, representative of two separate experiments. *$P$ < 0.05 compared with the saline group and **$P$ < 0.05 compared with the vehicle group (one-way ANOVA followed by Tukey's $t$-test).

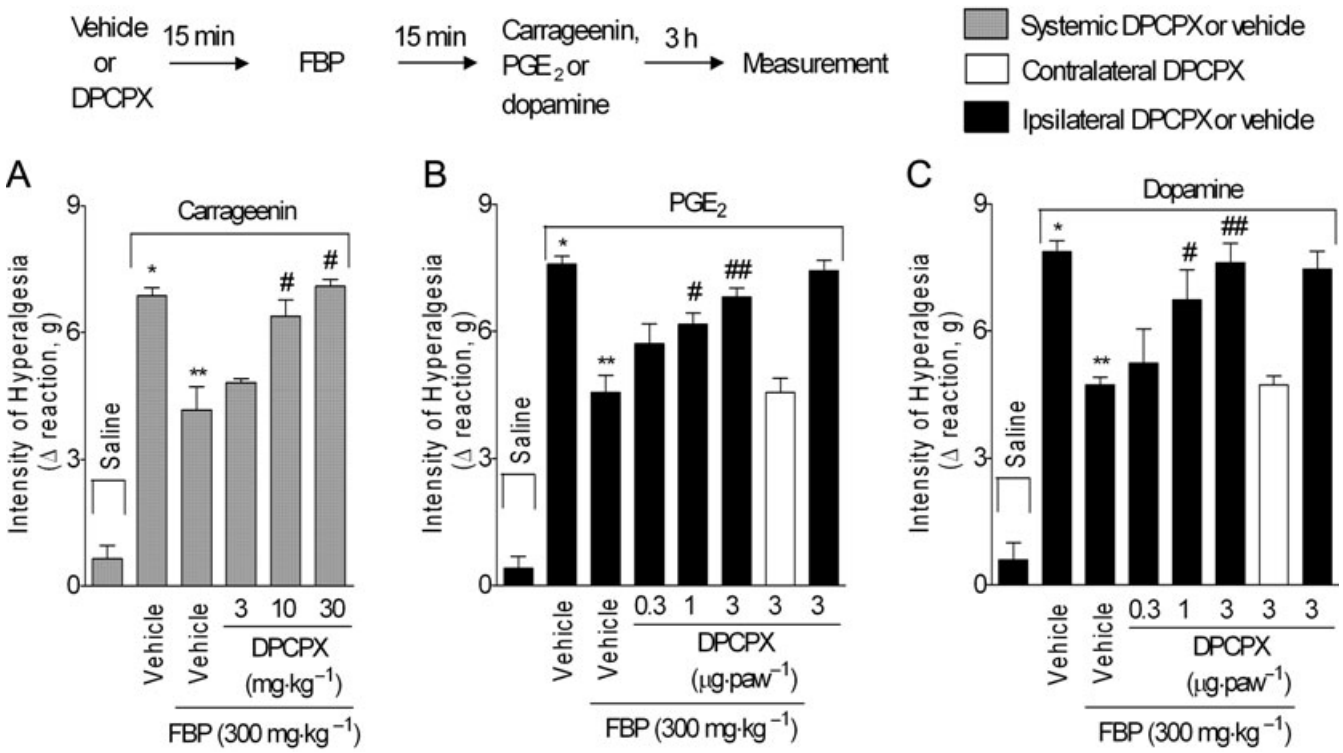


**Figure 4** DPCPX (8-cyclopentyl-1,3-dipropylxanthine) prevents the inhibition of mechanical hyperalgesia by D-Fructose-1,6-bisphosphate (FBP). Mice were treated with vehicle (Tween 80, 0.5% in saline) or DPCPX (adenosine $A_1$ receptor antagonist, 3–30 mg·kg$^{-1}$, s.c., 15 min) before FBP (300 mg·kg$^{-1}$, i.p., A) treatment, and after an additional 15 min, mice received intraplantar injection of carrageenin (100 μg per paw). In another set of experiments mice received local treatment with DPCPX (0.3–3.0 μg per paw, intraplantar, 15 min) before FBP (300 mg·kg$^{-1}$, i.p., B,C) treatment, and after additional 15 min mice received intraplantar injection of prostaglandin $E_2$ ($PGE_2$) (30 ng, B) or dopamine (10 μg, C). The intensity of hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-metre test. $n = 5$ mice per group per experiment, representative of two separate experiments. $^*P < 0.05$ compared with the saline group and $^{**}P < 0.05$ compared with the vehicle group; $^{\#}P < 0.05$ compared with the vehicle of DPCPX groups; $^{\#\#}P < 0.05$ compared with the vehicle of DPCPX 0.3 μg·paw$^{-1}$ groups. (one-way ANOVA followed by Tukey's *t*-test).

13 min and the internal standard theophylline (peak 2) a retention time of 27 min. Figure 5B shows the peaks obtained from blood samples of vehicle (saline)-treated mice with added adenosine (peak 1) and theophylline (peak 2), which presented the same retention times as observed in Figure 5A. In Figure 5C, the record shows blood samples collected 3 h after treatment with FBP (300 mg·kg$^{-1}$, i.p.), processed and analysed. Two peaks were detected: peak 1 of adenosine and peak 2 of theophylline (internal standard), with the same retention times observed in the control samples (Figure 5A,B). Another group of mice were treated with adenosine (100 mg·kg$^{-1}$, i.p.); blood samples were collected 3 h after the adenosine injection and processed as described. These samples showed a marked increase in the peak 1 (adenosine), and the retention times of peaks 1 and 2 (theophylline) were the same as presented in Figure 5A–C.

Figure 5E shows a standard curve for adenosine (0.2–6 μg·mL$^{-1}$) in HPLC assay from which a linear equation was obtained ($y = 1.02x - 0.0222$; $R^2 = 0.9999$) allowing the quantification of adenosine levels at 2 and 3 h after FBP (300 mg·kg$^{-1}$, i.p.) or adenosine (100 mg·kg$^{-1}$, i.p.) treatments. Summary data from these assays disclosed a significant increase in adenosine blood levels 2 and 3 h after FBP (Figure 5F) or adenosine (Figure 5G) treatment. FBP also induced an increase of adenosine blood levels 1 h after treatment (data no shown).

*Adenosine mimics the inhibition of inflammatory mechanical hyperalgesia by FBP*

Systemic treatment of mice with adenosine (100 mg·kg$^{-1}$, 15 min, i.p.) also reduced carrageenin- (Figure 6A), $PGE_2$- (Figure 6B) and dopamine- (Figure 6C) induced hyperalgesia. This antinociceptive effect of adenosine was prevented by both systemic (Figure 6A, 3–30 mg·kg$^{-1}$, i.p.) and local (Figure 6A,C, 0.3–3 μg per paw) treatments with DPCPX, suggesting a peripheral site of action. None of the DPCPX treatments altered the hyperalgesia induced by carrageenin, $PGE_2$ or dopamine *per se* (right bars of all panels in Figure 6A–C).

## Discussion

There is evidence showing that FBP presents anti-inflammatory activities in different models of inflammation (Planas *et al*., 1993; Mihas *et al*., 2003; Sola *et al*., 2003; Alves-Filho *et al*., 2004). In the present study, we have demonstrated that, besides these anti-inflammatory effects, FBP also exhibited an antinociceptive activity. This effect of FBP was not dependent on inhibition of cytokines production and neutrophil migration but more dependent on increased levels of adenosine, which in turn activated peripheral adenosine $A_1$ receptors and directly reduced inflammatory hyperalgesia.

In the experimental conditions used in this study, we previously demonstrated that carrageenin induces mechanical

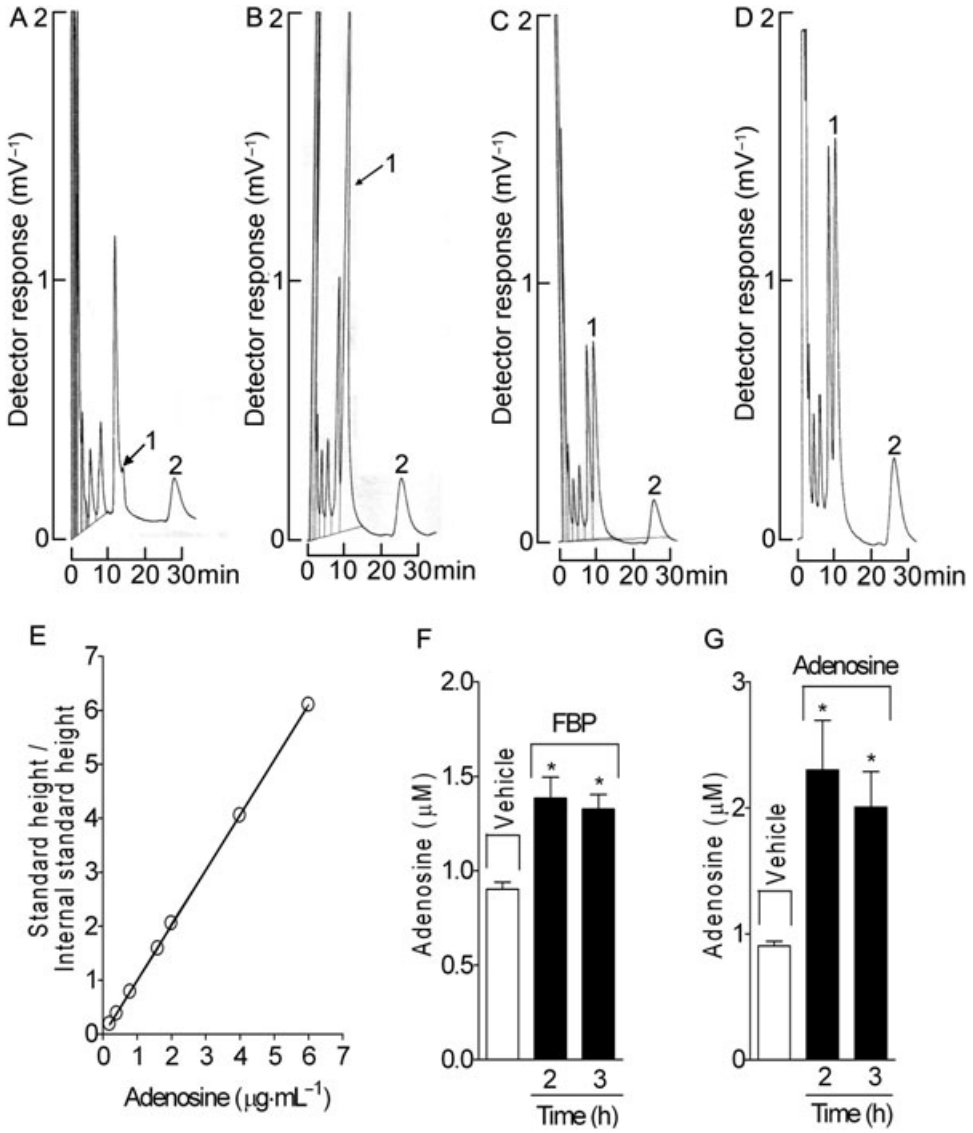


**Figure 5** D-Fructose-1,6-bisphosphate (FBP) increases adenosine plasma levels. (A–D) Representative chromatograms of plasma samples collected 3 h after treatment. (A) Retention time of endogenous adenosine levels (peak 1) and added theophylline (peak 2) in plasma samples from saline-treated mice. (B) Retention times and peak height of plasma samples added adenosine (1) and theophylline (2) from saline-treated mice. (C,D) adenosine peak (1) of plasma samples of mice treated with FBP (300 mg·kg$^{-1}$, i.p., C) or adenosine (100 mg·kg$^{-1}$, i.p., D) 3 h before. Theophylline was added as an internal standard (peak 2). (E) Standard curve of added adenosine in plasma samples. (F,G) Summary data for plasma levels of adenosine, 2 and 3 h after FBP or adenosine treatment respectively. Adenosine was quantified according to the equation ($y = 1.02x - 0.0222$; $R^2 = 0.9999$) obtained from the standard curve of adenosine (E). $n = 5$ mice per group per experiment, representative of two separate experiments. *$P < 0.05$ compared with the vehicle group (one-way ANOVA followed by Tukey's *t*-test).

hyperalgesia in mice by activating a sequential cytokine/chemokine cascade. This cascade starts with TNFα and CXCL1 production, and both induce the production of IL-1β that in turn activates cyclooxygenase to produce prostanoids. CXCL1 also triggers a parallel pathway to release sympathomimetic amines in mice. Prostanoids and sympathomimetic amines are ultimately responsible for nociceptor sensitization (Cunha *et al.*, 2005). Therefore, many drugs that inhibit cytokine/chemokine production, such as corticosteroids, thalidomide (Ribeiro *et al.*, 2000), pentoxifylline (Vale *et al.*, 2004) and natural products (such as sequiterpene lactones, Valerio *et al.*, 2007) are antinociceptive in inflammation models. *In vitro* treatment with FBP inhibited phytohemagglutinin-induced human T lymphocyte production of soluble IL-2 receptor and TNFα (Bordignon Nunes *et al.*, 2003). In agreement, FBP also inhibited concavalin A-induced splenocyte increase of mRNA for IL-1 and IL-6 (Markov *et al.*, 2002; Cohly *et al.*, 2004); LPS-(Hirokawa *et al.*, 2002; Tamaki *et al.*, 2002; Cuesta *et al.*, 2006) and D-galactosamin-induced TNFα production by Kupfer cells (Cuesta *et al.*, 2006). Thus, it was likely that FBP would diminish inflammatory mechanical hyperalgesia by inhibiting cytokine production. Unexpectedly, FBP treatment did not alter carrageenin-induced cytokine/chemokine production in our model, even though cytokine modulation is involved in the antinociceptive mechanism of action of FBP. A major difference between our and previous experimental conditions is the *in vivo* approach we used. Additional components that taken together might be responsible for these different

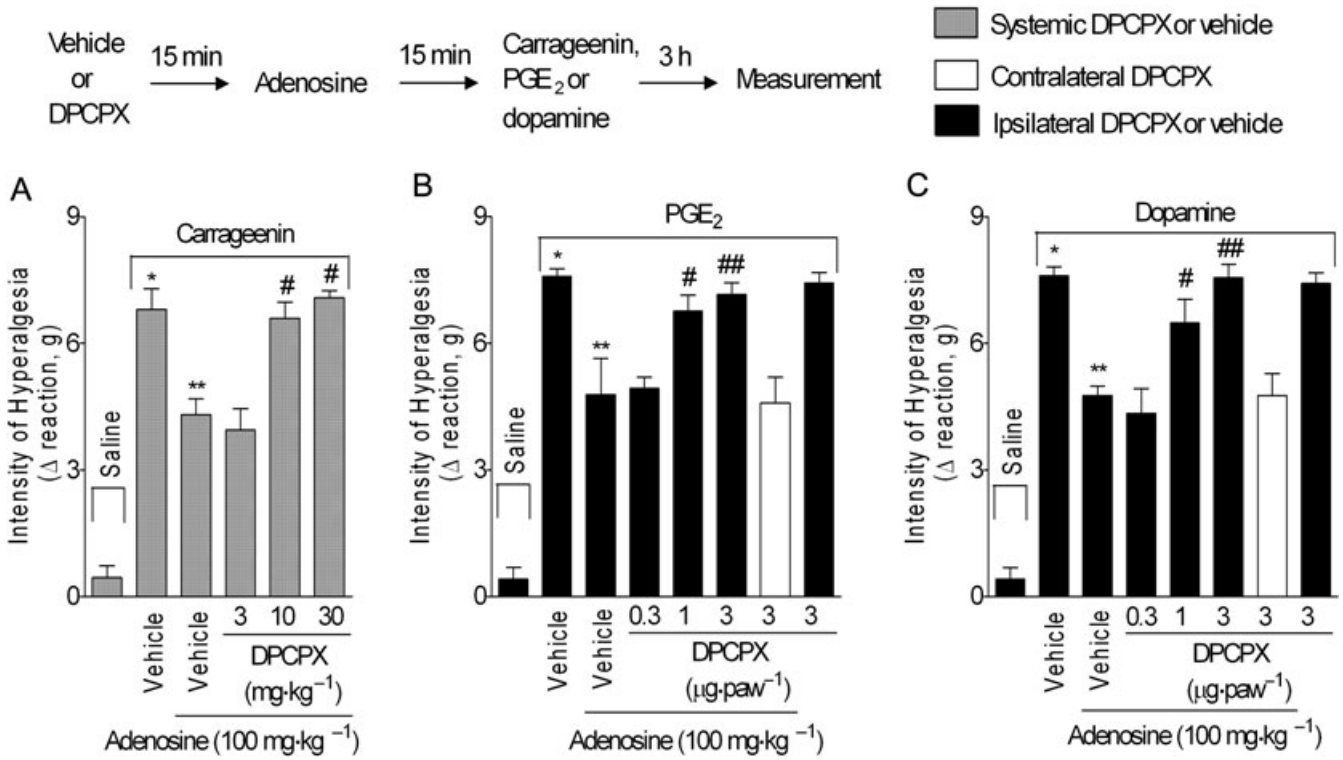


**Figure 6** Adenosine, acting on adenosine $A_1$ receptors, mimics inhibition of inflammatory mechanical hyperalgesia by D-Fructose-1,6-bisphosphate (FBP). (A) Mice were treated with vehicle (Tween 80, 0.5% in saline) or DPCPX (8-cyclopentyl-1,3-dipropylxanthine) (adenosine $A_1$ receptor antagonist, 3–30 mg·kg$^{-1}$, s.c., 15 min) before adenosine (100 mg·kg$^{-1}$, i.p.) treatment, and after additional 15 min mice received intraplantar injection of carrageenin (100 μg per paw). (B,C) In another set of experiments mice received local treatment with DPCPX (0.3–3.0 μg per paw, intraplantar, 15 min) before adenosine (100 mg·kg$^{-1}$, i.p.) treatment, and after additional 15 min mice received intraplantar injection of prostaglandin $E_2$ ($PGE_2$) (30 ng, B) or dopamine (10 μg, C). The intensity of hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-metre test. $n = 5$ mice per group per experiment, representative of two separate experiments. $^*P < 0.05$ compared with the saline group and $^{**}P < 0.05$ compared with the vehicle group; $^{\#}P < 0.05$ compared with the vehicle of DPCPX groups; $^{\#\#}P < 0.05$ compared with the DPCPX 0.3 μg·paw$^{-1}$ groups. (one-way ANOVA followed by Tukey's *t*-test).

responses in cytokine/chemokine production are the different stimuli, pharmacokinetics and cellular target(s) of FBP. While cytokine/chemokine generation is important to the development of inflammatory hyperalgesia by triggering the production of directly acting mediators (prostaglandins and sympathomimetic amines), hyperalgesia induced by cytokine/chemokines is also strongly dependent on neutrophil recruitment (Levine *et al.*, 1984; Lavich *et al.*, 2006; Cunha *et al.*, 2008a; Guerrero *et al.*, 2008; Ting *et al.*, 2008; Verri *et al.*, 2009). Because FBP inhibited the hyperalgesia induced by TNFα, IL-1β and CXCL1, another target for FBP could be inhibition of neutrophil migration. In contrast to this assumption, FBP did not affect carrageenin-induced neutrophil migration as determined by myeloperoxidase activity in the paw tissues, suggesting that, at the antinociceptive dose used in this study, FBP did not impair neutrophil migration.

Prostaglandin $E_2$ and dopamine acting on their receptors present in the nociceptors' membrane trigger a different intracellular signalling pathway, which promotes nociceptor sensitization. The fact that FBP treatment also inhibited $PGE_2$- and dopamine-induced hyperalgesia, suggests that FBP directly reduces nociceptor sensitization or even that FBP can induce an endogenous mediator with this action. One possible endogenous mediator involved in the activity of FBP is adenosine (Akimitsu *et al.*, 1995; Sola *et al.*, 2003). Indeed, adenosine has been reported to mimic the anti-inflammatory effect of FBP on microvascular permeability, myeloperoxidase activity and ischaemia reperfusion injury (Akimitsu *et al.*, 1995; Sola *et al.*, 2003). Furthermore, increased metabolism of adenosine can suppress the anti-inflammatory effect of FBP (Sawynok *et al.*, 1998; Sola *et al.*, 2003). Here, we provide more evidence that adenosine is crucial for the pharmacological effect of FBP. For instance, the treatment of mice with a selective antagonist of adenosine $A_1$ receptors prevented the antinociceptive effect of FBP. The participation of adenosine $A_1$ receptors in the antinociceptive effect of FBP seems to be mediated, at least in part, by peripheral receptors, as it was blocked by the local, intraplantar injection of the antagonist. This finding is in line with the present data showing that direct administration of adenosine $A_1$ receptor agonist in the rat paw blocks mechanical hyperalgesia induced by $PGE_2$. Although we did not investigate the molecular mechanism involved in the peripheral antinociceptive action of adenosine $A_1$ receptor activation, there is evidence suggesting that it is mediated by inhibition of cAMP formation, which is an important step in nociceptor sensitization and consequently in the establishment of inflammatory hyperalgesia (Levine *et al.*, 1993; Rang *et al.*, 1994; Sawynok, 1998). However, our findings cannot exclude the participation of adenosine receptors present in the CNS, and the effects of adenosine could be mediated by adenosine $A_1$ receptors present in the spinal cord, whose activation is known to produces antinociception in

different inflammatory models (Jurna, 1984; Sawynok *et al*., 1986; Santicioli *et al*., 1992; Santicioli *et al*., 1993; Li and Perl, 1994; Mauborgne *et al*., 2002; Schmidt *et al*., 2009).

The pharmacological evidence that adenosine through activation of adenosine $A_1$ receptor mediates the antinociceptive action of FBP was supported by the finding that the *in vivo* administration of FBP increased the levels of adenosine in blood. Although we do not know how FBP could raise the blood adenosine levels, the following data could explain this finding: (i) FBP can be absorbed by cells through a yet undetermined transporter, osmotic gradient or be carried together with other molecules (Hardin and Roberts, 1994; Ehringer *et al*., 2000); (ii) once inside the cell, the unexpected FBP increase can deregulate the glycolytic pathway because this intermediate is intimately linked to the regulation of many metabolic pathways (Kirtley and McKay, 1977; Nuutinen *et al*., 1991); and (iii) FBP is a step after enzymes such as hexokinase and phosphofructokinase that are regulatory points of the pathway, and before pyruvate kinase of which FBP is an allosteric activator (Taylor and Bailey, 1967; Bailey *et al*., 1968; Irving and Williams, 1973). Therefore, it is possible that FBP causes an increase in the intermediates of glycolysis, which induces an efflux of AMP from the cell. This AMP can be hydrolysed to adenosine in the extracellular environment, which would then be available to bind to adenosine $A_1$ receptors.

In conclusion, the present study demonstrates that: (i) FBP reduced inflammatory hyperalgesia; (ii) the antinociceptive effect of FBP did not depend on inhibition of cytokine production; (iii) FBP and adenosine inhibited the hyperalgesia with a similar profile; (iv) FBP increased blood levels of adenosine, explaining the similarity of action between FBP and adenosine; and (v) the antinociceptive effect of FBP and adenosine depended on activation of $A_1$ receptors. Thus, the present study elucidates a novel pharmacological activity of FBP, suggesting that it merits further clinical investigation as a possible analgesic drug.

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