

BACKGROUND: Studies on peripheral benzodiazepine receptor function have yielded a diverse list of activities of which the anti-inflammatory effects need to be further examined.

Aims: To evaluate the role of steroids, nitric oxide and adenosine-deaminase in the anti-inflammatory effect of PK11195.

Methods: Pleurisy was induced by intrapleural injection of carrageenan in mice pre-treated or not with PK11195. Leukocytes, exudation, adenosine-deaminase (ADA) activity and nitric oxide (NO) level were measured. Steroid involvement was evaluated by pre-treatment with D,L-aminogluthetimide before PK11195.

Results: Leukocytes, exudation and NO levels were reduced by PK11195 in the early (4 h) phase. In the late (48 h) phase, PK11195 decreased leukocytes and ADA activity. D,L-aminogluthetimide reversed the effect of PK11195 on exudate (4 h), as well as total and differential leukocytes and NO levels (48 h).

Conclusions: Steroids, NO and ADA are implicated in the anti-inflammatory action of PK11195.

Key words: PK11195, Pleurisy, Nitric oxide, Steroid Synthesis

Involvement of steroids in anti-inflammatory effects of PK11195 in a murine model of pleurisy

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Introduction

The peripheral benzodiazepine receptor (PBR) is an 18 kDa protein localised on the mitochondrial outer membrane, where it is associated with at least two other proteins: the voltage-dependent anion channel protein, and the adenine nucleotide carrier.^{1,2} PK11195 [1-(2-chlorophenyl)-N-methyl-N-(1-methylpropyl)-3-isoquinoline carboxamide] is one of the most widely studied specific ligands for the PBR. Only the 18 kDa subunit carries PK11195 binding domains.³ The PBR is expressed in many tissues and abundantly in classical steroid-producing cells.^{4,5} PBR ligands stimulate steroid synthesis not only in endocrine tissues such as adrenal glands, placenta, testes and ovaries, but also in the liver, glia and Schwann cells by enhancing the translocation of cholesterol from outer to inner mitochondrial membranes, the rate-limiting step in steroidogenesis.^{6–12} Furthermore, the presence of PBRs in other tissues in which steroidogenesis occurs, such as the pancreas and the heart, suggests a role for these receptors in steroid biosynthesis in different parts of the body.^{13,14}

The PBR is widely distributed in the immune system organs such as the spleen, thymus, intestine and lymph nodes and in various types of human blood cells,^{15,16} presupposing an involvement of this receptor in inflammatory and immune processes.

In a previous study, it was demonstrated that a long pre-treatment time (24 h) with PK11195 had an inhibitory effect on neutrophil influx, without inhibition of exudation in the inflammatory response 4 h after carrageenan injection in a model of mouse pleurisy.¹⁷ Here, we report that a shorter pre-treatment time (1 h) with PK11195 affects pleural exudate formation and cellular infiltration in both early (4 h) and late (48 h) inflammatory phases of carrageenan-induced pleurisy in mice. The participation of steroids in these anti-inflammatory PK11195 effects was examined by using D,L-aminogluthetimide, a potent inhibitor of P450_{scc}, the enzyme responsible for the transformation of cholesterol into pregnenolone.¹⁸

Adenosine has signaling, cytoprotective and anti-inflammatory roles depending on the receptor subtype and the levels of adenosine available.¹⁹ Thus, the subsequent interaction of adenosine with adenosine type 2 receptors may inhibit, among others, the expression of CD11b/CD18 molecules in leukocytes that promote and facilitate cell migration.²⁰ In airways, A_{2A} receptors are present on many immunoinflammatory cells. Moreover, these receptors are implicated in the suppression of degranulation of neutrophils, mast cells, monocytes and T lymphocytes.^{21–23} Adenosine-deaminase (ADA) is an enzyme that controls the concentration of adenosine in organs and cells. ADA activity is significantly increased in pleurisy induced by carrageenan.²⁴ Several studies have shown that ADA is able to

suppress the effects of the released adenosine.²⁵ On the other hand, nitric oxide (NO) seems to play an important role in the carrageenan-induced inflammatory response. Thus, the lack of NO generation due to the absence of inducible nitric oxide synthase (iNOS) expression produces a significant reduction of the pleural exudate and the number of emigrated polymorphonuclear cells in carrageenan-induced pleurisy.²⁶

Therefore, to better understand the mechanisms of the observed anti-inflammatory effect of PK11195, we analysed whether this involves steroid synthesis as the underlying mechanism and the regulation of adenosine and nitric oxide production in the carrageenan-induced pleurisy model.

Methods

The experiments were performed with the approval of the Committee for the Use of Animals in Experiments of the Universidade Federal de Santa Catarina (Brazil).

Animals

Non-fasted adult Swiss mice of both sexes (18–22 g; $n = 4-6$), aged 1 month, were used throughout the experiments. They were housed in accordance with institutional animal care requirements (temperature $21 \pm 2^\circ\text{C}$, under a light/dark cycle of 12 h) and fed freely on standard rodent chow and water.

Drugs

PK11195 from Sanofi-Synthélabo Recherche (Labège, France) was dissolved in absolute ethanol and diluted with phosphate-buffered saline (PBS) to the appropriate concentrations. The maximum concentration of ethanol injected into the animal was 1%. The vehicle utilised in control animals was 1% ethanol in PBS. D,L-Aminoglutethimide, carrageenan (degree IV), sodium azide, *o*-dianisidine 2HCl (3,3'-dimethoxybenzidine), and human polymorphonuclear leukocyte myeloperoxidase were purchased from Sigma (St Louis, MO, USA). The D,L-aminoglutethimide was dissolved in dimethylsulfoxide (DMSO) and diluted in PBS to the appropriate concentration. The maximum concentration of DMSO injected into the animal was 10% and the vehicle utilised in control animals was 10% DMSO in PBS. Neither 1% ethanol nor 10% DMSO affected the inflammatory parameters evaluated 4 and 48 h after pleurisy induction. The other drugs were purchased, stocked and diluted as indicated: heparin (Liquemine®; Roche, São Paulo, SP, Brazil),

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, NH_3SO_4 , nitroprussiate (Montedison, São Paulo, SP, Brazil), adenosine (Fluka, Ronkonkoma, NY, USA), alkaline buffer, hydrogen peroxide (30%), disodium *N*-hydrogen phosphate-12-hydrated, sulfanilic acid, *N*-(1-naphyl)-ethylenodiamine dihydroxide, zinc-sulfate-7-hydrated, sodium phosphate, formamide (Merck, São Paulo, SP, Brazil), ammonium formate (Riedel-de Hën, Seelze, Germany), phenol (Biotech, São Paulo, SP, Brazil). NaCl (0.9%), Evans Blue and May–Grunwald–Giemsa dyes from different commercial sources. PBS (Merck) was previously prepared and maintained in the refrigerator. All drugs were kept in siliconised plastic tubes at -20°C . On the day of the experiments, the drugs were diluted to the desired concentration with NaCl (0.9%) at room temperature.

Experimental design

Carrageenan-induced pleurisy

As previously described,^{27,28} pleurisy was induced by a single intrapleural injection of 0.1 ml of carrageenan (1%). Since the pleurisy caused by carrageenan exhibits a biphasic response (4 and 48 h), these two interval points were chosen to analyse the studied parameters.

After killing the animals with an overdose of ether, the thorax was opened and the pleural cavity was washed with 1.0 ml of sterile PBS containing heparin (20 IU/ml). Several samples of the pleural lavage were collected for further determinations of ADA activity,²⁹ exudate levels and nitric oxide concentrations,³⁰ as well as total and differential leukocyte contents. Total leukocytes were performed on an automatic counting machine (Beckman Coulter Inc., Brea, CA, USA), while cytospin preparations of pleural wash were stained with May–Grunwald–Giemsa for the differential count, which was performed under an oil immersion objective. All animals, except in the experiments that analysed the enzyme activity or nitric oxide levels, were previously challenged (1 h) with a solution of Evans blue dye (25 mg/kg, intravenously) in order to evaluate the degree of exudation in the pleural cavity. A sample (500 μl) of the fluid collected from the pleural cavity was stored in a freezer (-20°C) to further determine the concentration of Evans blue dye. To this end, on the day of the experiments, a batch of samples was thawed at room temperature and the amount of dye was estimated by colorimetry using an Elisa plate reader (Organon Teknika, Roseland, NJ, USA) at 600 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01–50 $\mu\text{g/ml}$.

Evaluation of the effects of PK11195 on exudation and cellular infiltration in carrageenan-induced pleurisy

For dose–response studies, a group of animals was treated 1 h before pleurisy induction with different doses of PK11195 [0.01–1.0 mg/kg, intraperitoneally (i.p.)] or vehicle, and the inflammatory parameters (leukocytes and exudation) were analysed 4 and 48 h after carrageenan injection. In another set of experiments, animals were pre-treated with one dose of PK11195 (0.1 mg/kg, i.p.) or vehicle at different times (0.5–4 h) and the same inflammatory parameters were evaluated 4 and 48 h after pleurisy induction. For the time course of the later (48 h) phase, further groups of animals were also treated with a single injection (1 h) or with two injections of PK11195 (0.1 mg/kg, i.p.) or vehicle with a 12 h interval (the first dose of PK11195 at 1 h before carrageenan and the second dose 12 h after the first one).

Evaluation of the participation of steroids in the fluid leakage and cell influx inhibition by PK11195

A group of animals was pre-treated (0.5 h) with 10 mg/kg of D,L-aminoglutethimide before 0.1 mg/kg (i.p.) of PK11195 or vehicle, 1 h before carrageenan administration. Thereafter, the pleural lavage samples for the leukocytes counts and quantification of exudation were evaluated 4 and 48 h after pleurisy induction.

Determination of nitrate/nitrite concentrations

Mice treated with PK11195 or vehicle (0.1 mg/kg, i.p.) 1 h prior to pleurisy induction or twice with a 12 h interval prior to pleurisy induction were utilised in order to evaluate the action of PK11195 in the early (4 h) and late (48 h) phases of pleurisy upon nitrate/nitrite levels, an indicator of NO synthesis in samples of pleural lavage fluid.

Samples of the pleural lavage obtained from control and treated animals that did not receive Evans blue dye injection were collected, separated and stored at -20°C , and the nitrate/nitrite levels were determined by the Griess reaction as previously described by Saleh et al.³⁰

Evaluation of the effect of PK11195 on the adenosine-deaminase activity in pleurisy

ADA activity was measured 4 and 48 h after pleurisy induction, according to the method developed by Giusti and Galanti,²⁹ in samples of pleural lavage from animals treated with 0.1 mg/kg of PK11195 (i.p.) or vehicle given at 1 h or twice with a 12 h interval. Using conventional reagents, the enzyme concentration was estimated by means of colorimetric mea-

surement (absorbency at 630 nm) on an Elisa plate reader (Organon Teknika).

One unit of ADA is equivalent to the amount of enzyme required to release 1 mmol of ammonia/min. Results are expressed as units per litre. A detailed description of this assay has been published previously by Fröde and Medeiros.²⁴

Statistical analysis

Data are reported as the mean \pm standard error of the mean. Differences between groups were determined by analysis of variance complemented with Dunnett's and/or Student's tests. $p < 0.05$ was considered indicative of significance.

Results

Determination of the PK11195 effects on leukocytes and exudation in carrageenan-induced pleurisy

The time course studies indicated that only mice treated with PK11195 0.5 and 1 h prior to the pleurisy induction showed a significant inhibition of leukocyte recruitment (percentage inhibition: 0.5 h, 33 ± 14 and 1 h, 32 ± 9 , respectively; $p < 0.05$) (Fig. 1). This effect was due to neutrophils influxes (percentage inhibition: 0.5 h, 37 ± 15 and 1 h, 34 ± 10 , respectively; $p < 0.05$) (Fig. 1, inset). Furthermore, Fig. 1 also shows that only the treatment of animals with PK11195 at 1 h caused a significant inhibition in exudate levels (percentage inhibition, 37 ± 7 ; $p < 0.01$).

In a previous study it was demonstrated that mice treated with PK11195 (0.1 mg/kg) at 24 h prior to pleurisy induction by carrageenan showed a significant reduction in neutrophils, but no decrease in the exudate levels.¹⁷ Thus, to permit the study of the action of PK11195 on both cell recruitment and exudation we chose to pre-treat animals with PK11195 at 1 h before pleurisy induction for the dose–response studies.

Figure 2 shows that PK11195 (0.1–1.0 mg/kg, i.p.) administered 1 h before carrageenan inhibited significantly the leukocyte influx in the early (4 h) phase of pleurisy induction (percentage inhibition: 0.1 mg/kg, 32 ± 9 ; 0.5 mg/kg, 34 ± 7 ; and 1.0 mg/kg, 26 ± 9 ; $p < 0.05$). The lowest dose of PK11195 (0.01 mg/kg) had no significant effect on leukocyte influx. Figure 2 (inset) shows that this inhibitory effect was due mainly to neutrophil influxes (percentage inhibition: 0.1 mg/kg, 34 ± 10 ; 0.5 mg/kg, 33 ± 7 ; and 1.0 mg/kg, 25 ± 9 ; $p < 0.05$). Furthermore, the dose of 0.5 mg/kg, i.p., 1 h before was also effective in inhibiting mononuclears (percentage inhibition, 55 ± 7 ; $p < 0.05$).

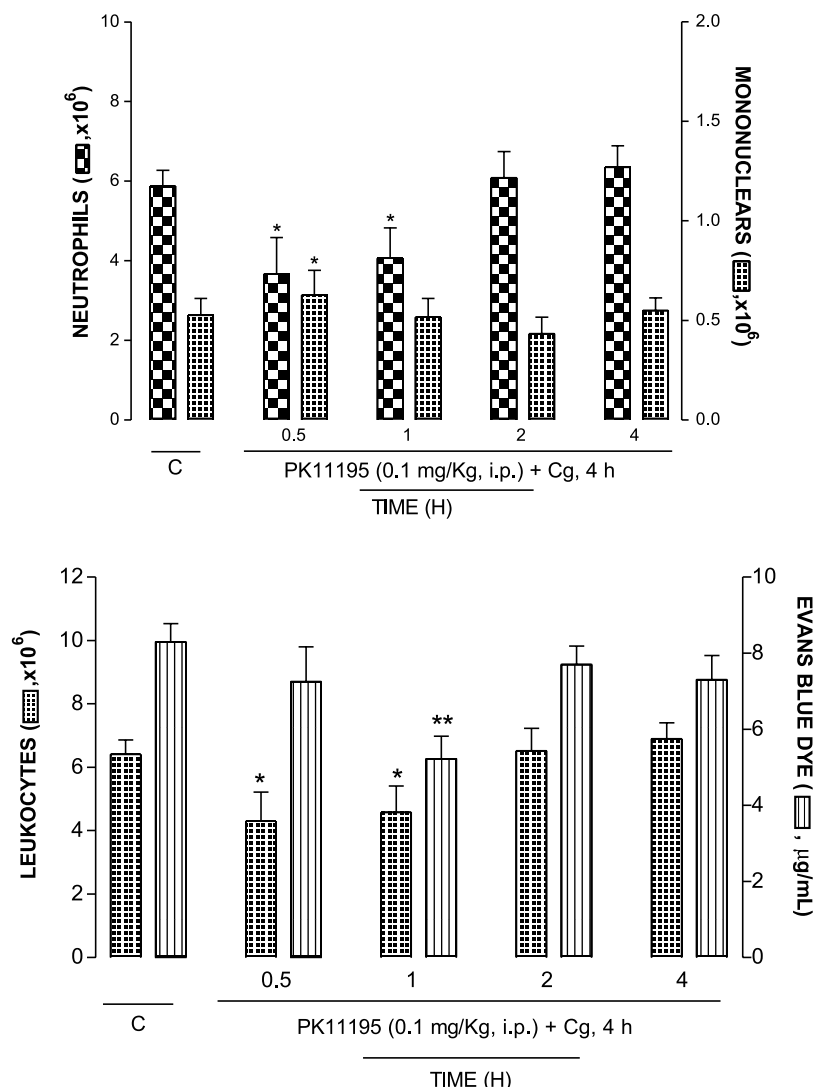


FIG. 1. Effects of PK11195 administered 0.5–4 h before carrageenan (Cg, 1%/cav.) on the early (4 h) phase of the mouse pleurisy induced by Cg. Leukocytes and exudation levels. Inset: Mononuclear and neutrophil cells, under the same conditions. C, Control responses in animals only treated with Cg. Each column represents the mean of four to six animals, and the vertical bars represent the standard error of the mean. * $p < 0.05$ and ** $p < 0.01$.

An unexpected increase in mononuclear influx was observed with 0.01 mg/kg (percentage enhancement, 90 ± 3 ; $p < 0.05$). On the other hand, only 0.1 mg/kg of PK11195 produced a significant inhibition of exudation level ($p < 0.01$) (Fig. 2).

The time course studies (Fig. 3) carried out in the later (48 h) phase, however, indicated that pre-treatment with PK11195 at 2 and 4 h produces a significant enhancement of leukocyte recruitment (percentage enhancement: 2 h, 35 ± 6 and 4 h, 79 ± 6 ; $p < 0.01$). Figure 3 (inset) shows that this effect was due mainly to an enhancement of neutrophils (percentage enhancement: 2 h, 354 ± 10 and 4 h, 391 ± 12 ; $p < 0.01$). However, mononuclear cells were significantly fewer at 1, 2 and 4 h (percentage reduction: 1 h, 55 ± 12 ; 2 h, 75 ± 3 ; and 4 h, 22 ± 8 ; $p < 0.01$). No change in exudate levels was observed

with these pre-treatment times (data not shown). In another group that received one first dose of PK11195 at 1 h before carrageenan and a second dose 12 h after the first one, a significant decrease in the leukocyte infiltration was observed (percentage decrease, 50 ± 3 ; $p < 0.01$) (Fig. 3) with no change in exudate levels (data not shown). This leukocyte inhibition was due to reduced mononuclear cell influx (percentage inhibition, 74 ± 2 ; $p < 0.01$) (Fig. 3, inset).

Determination of the participation of steroids in the fluid leakage and cell influx inhibition by PK11195

Figure 4A shows that treatment of animals with D,L-aminoglutethimide produced a reversal of the inhi-

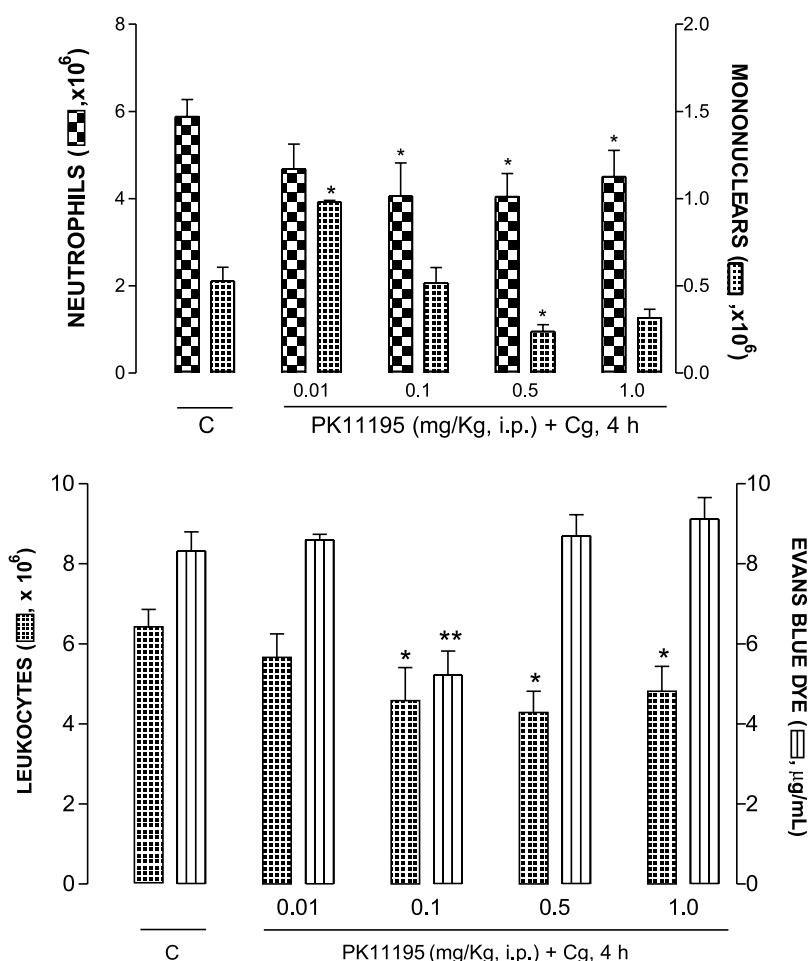


FIG. 2. Effects of different doses of PK11195 (0.01–1.0 mg/kg, i.p.) administered 1 h prior to carrageenan (Cg, 1%/cav.), on the early (4 h) phase of the mouse pleurisy induced by Cg. Leukocytes and exudation levels. Inset: Mononuclear and neutrophil cells, under the same conditions. C, Control responses in animals only treated with Cg. Each column represents the mean of four to six animals, and the vertical bars represent the standard error of the mean. * $p < 0.05$ and ** $p < 0.01$.

bitory effect of PK11195 on exudate levels, with no change in neutrophil influxes in the early (4 h) phase of pleurisy ($p < 0.01$) (Fig. 4A, inset). In the later (48 h) phase, nevertheless, D,L-aminogluthetamide was able to reverse not only the inhibitory effect of PK11195 on mononuclear influx, but also caused the accumulation of a volume of pleural exudate ($p < 0.01$) (Fig. 4B and 4B, inset). Exudate levels and leukocytes influx were not affected by D,L-aminogluthetamide without PK11195 (results not shown).

Evaluation of effect of PK11195 on the activities of NO and ADA in pleurisy

The level of nitrate/nitrite in the lavage fluid from animals treated with PK11195 significantly decreased in the early (4 h) phase of pleurisy (percentage inhibition, 47 ± 6 ; $p < 0.01$) (Table 1). However, PK11195, was ineffective in inhibiting nitrate/nitrite levels in the later (48 h) phase of pleurisy (Table 1).

PK11195, when administered in two doses of 0.1 mg/kg, i.p., with a 12 h interval, only inhibited the

ADA activity in the late (48 h) phase of pleurisy (percentage inhibition, 58 ± 10 ; $p < 0.01$) (Table 1).

Discussion

Our results demonstrate that in a pleurisy model pre-treatment of mice with PK11195 at 0.5 and 1 h before carrageenan produced an important anti-inflammatory action in the early (4 h) phase due mainly to inhibition of neutrophil migration and fluid leakage formation. On the late (48 h) phase, however, only treatment with two injections of PK11195, a first 1 h before carrageenan and a second 12 h after the first one, was effective in inhibiting the leukocyte influx due to mononuclear cells without changes in exudate. These results are in accordance with a previous study, where it was observed that the anti-inflammatory action of PK11195, in a model of acute inflammation, disappeared 48 h after treatment.³¹

It has been shown that PK11195 administered 24 h before carrageenan in a pleurisy model exerted an inhibitory effect on neutrophil influx, but no change

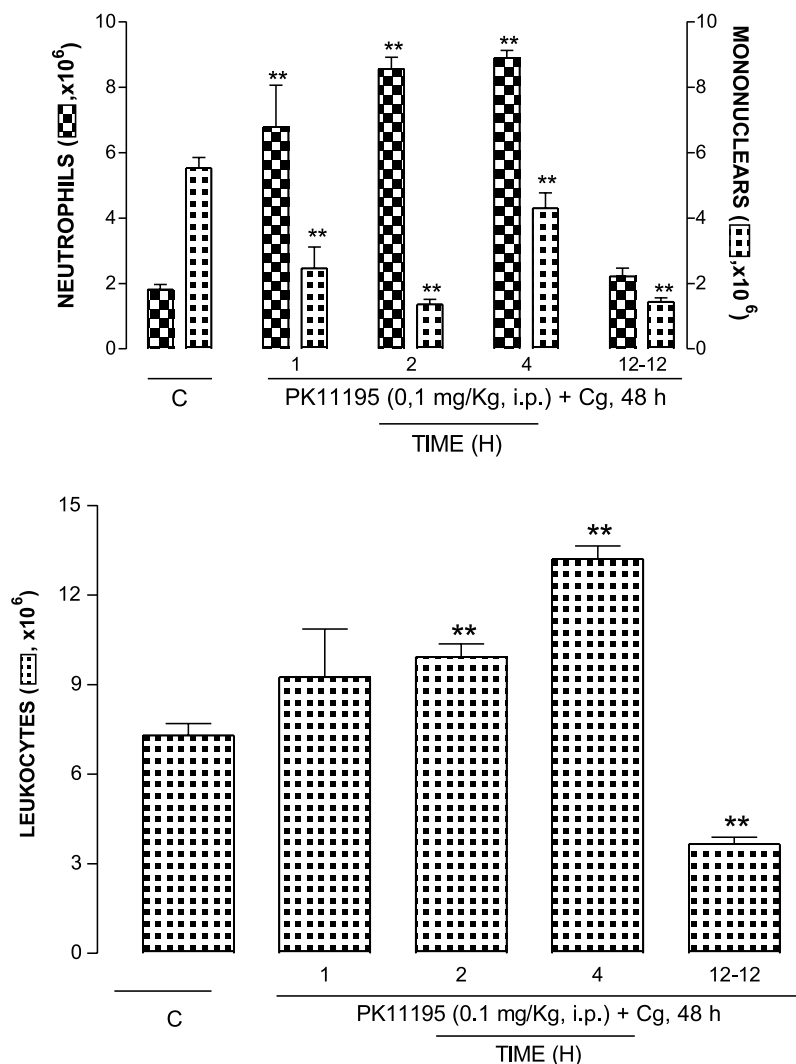


FIG. 3. Effects of PK11195 (0.1 mg/kg, i.p.), administered 1–4 h before carrageenan (Cg, 1%/cav.) or twice with a 12 h interval, on the late (48 h) phase of the mouse pleurisy induced by Cg. Leukocytes and exudation levels. Inset: Mononuclear and neutrophil cells, under the same conditions. C, Control responses in animals only treated with Cg. Each column represents the mean of four to six animals, and the vertical bars represent the standard error of the mean. ** $p < 0.01$.

in exudate levels in the early phase of pleurisy.¹⁷ Thus, in this study, to characterise the role of the PBR in both end points of the inflammatory process (exudate formation and neutrophils infiltration) in the early (4 h) phase of pleurisy, we chose to pre-treat animals with PK11195 at 1 h before carrageenan. As the single injections of PK11195 at all times of pre-treatment analysed were not very effective in inhibiting either total cell recruitment or exudate, animals treated twice with PK11195 at 12 h time intervals were utilised for the study of the mechanisms of action of PK11195 in the late phase of pleurisy.

The mechanism responsible for the anti-inflammatory action of PK11195 is not yet understood clearly. PK11195 binding is increased in several pathological situations where an inflammatory process is present.³² Pre-treatment with PK11195 prevents the neurodegenerative effects, and increases PK11195 binding protein levels in the hippocampus of animals treated with kainic acid.³³ The PBR density was

greatly increased in rat sciatic nerve degeneration, and the administration of Ro5-4864 (i.p.) induced a significant increase in pregnenolone levels in sciatic nerve and plasma.¹² These results suggest that glucocorticoid synthesis could be involved in PBR ligands effects. Here, we confirmed the participation of glucocorticoids in the anti-inflammatory action of PBR activation by the utilisation of an inhibitor of pregnenolone synthesis. Thus, D,L-aminogluthetamide reversed the inhibitory effect of PK11195 on exudation, but not on neutrophil influx to the mouse pleural cavity in the early (4 h) phase of the inflammatory response induced by carrageenan. Furthermore, glucocorticoids are also implicated in the inhibitory effect of PK11195 on mononuclear cell influx observed in the late (48 h) phase of pleurisy.

Very few studies exist on the mediator implicated in carrageenan-induced mouse pleurisy, but bradykinin (BK) and its degradative metabolite des-Arg9-Bk seem to have an important role in fluid leakage

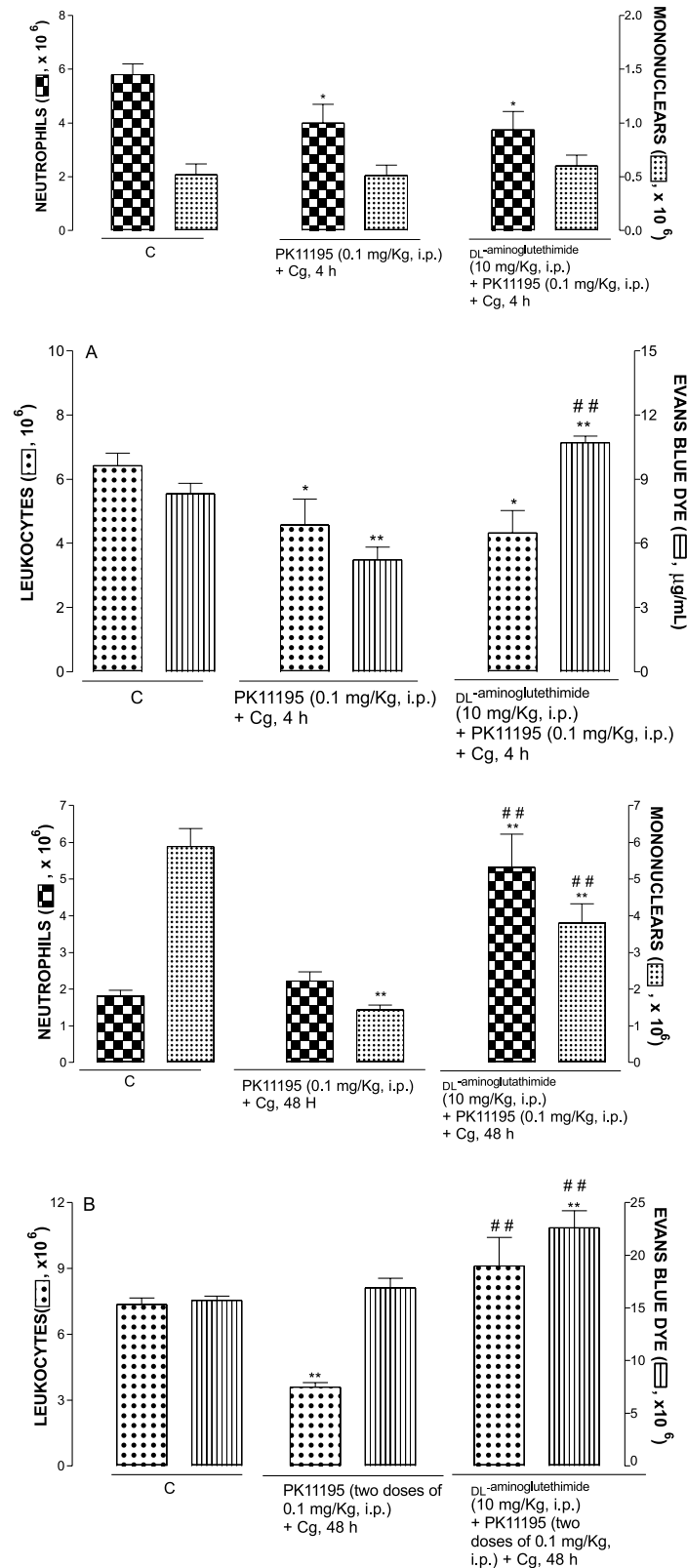


FIG. 4. Effects of D,L-aminogluthetimide and PK11195 association on early (4 h) or late (48 h) phases of the mouse pleurisy induced by carrageenan (Cg, 1%/cav.). The animals were pre-treated (0.5 h) with D,L-aminogluthetimide (10 mg/kg, i.p.) before PK11195 (0.1 mg/kg, i.p.) and then mouse pleurisy was induced by Cg. (A) Leukocytes and exudation levels in the early (4 h) phase of the inflammatory response. (B) Leukocytes and exudation levels in the late (48 h) phase of the inflammatory process. Insets: Mononuclear and neutrophil cells, under the same conditions. C, Control responses in animals only treated with Cg. Each column represents the mean of four to six animals, and the vertical bars represent the standard error of the mean. * $p < 0.05$ and ** $p < 0.01$, Cg versus PK11195; ## $p < 0.01$, PK11195 versus D,L-aminogluthetimide plus PK 11195.

Table 1. Effect of the pre-treatment of the animals with PK11195 upon nitrate/nitrite and adenosine-deaminase levels on the early (4 h) and late (48 h) phases of the inflammatory reaction induced by carrageenan in the mouse pleural cavity

Group	Pleurisy 4 h		Pleurisy 48 h	
	Nitrate/nitrite (μM)	Adenosine-deaminase (IU/ml)	Nitrate/nitrite (μM)	Adenosine-deaminase (IU/ml)
Saline treated ^a	29 \pm 0.4	1.9 \pm 0.2	2.9 \pm 0.4	1.9 \pm 0.2
Carrageenan treated ^a	20.0 \pm 1.3	5.0 \pm 0.7	10.8 \pm 0.8	6.9 \pm 0.4
PK11195 ^b (0.1 mg/kg)	9.4 \pm 1.1**	4.3 \pm 1.2		
PK11195 ^c (0.1 mg/kg)	–	–	9.2 \pm 0.9	3.3 \pm 0.5**

The parameters were analysed 4 and 48 h after the administration of either carrageenan or buffered-saline in the pleural cavity. Values represent the mean \pm standard error of the mean ($n = 4-6$ animals per group). Statistical differences determined by analysis of variance, complemented with Dunnett's test are indicated: ** $p < 0.01$.

^a Treatment given to the pleural cavity.

^b Treatment given by i.p. route, 1 h before carrageenan.

^c Treatment given by i.p. route with two doses of PK11195 of 0.1 mg/kg with a 12 h interval.

formation and leukocyte migration. Two subtypes of bradykinin receptors have been identified, B₁ and B₂.³⁴ The bradykinin B₂ receptor is expressed constitutively in several tissues and is considered to mediate the majority of bradykinin effects such as vasodilatation and increased vascular permeability. BK stimulates the synthesis of IL-1, IL-2, and IL-6 from lung strip explants, an effect susceptible to blockade by a B₂ BK receptor antagonist.³⁵ In lung fibroblasts BK induces synthesis of IL-6 and IL-8 via the B₂ receptor.³⁶ The bradykinin B₁ receptor, however, is not widely expressed in normal tissues, but is highly inducible by inflammatory mediators as IL-1. The interaction between interleukin-1 and B₁ receptors was clearly established in murine models of inflammation where neutrophil accumulation induced by interleukin-1 was due to up-regulation of B₁ receptors whose activation causes the release of substance P and calcitonin gene-related peptide from sensory C afferent fibres.^{37,38} On the other hand, B₁ receptor agonists can mediate the induction of B₁ receptors in lung fibroblasts by a distinct but synergic mechanism involving IL-1. Thus, the p38 mitogen-activated protein kinase pathway is implicated in B₁ agonist-promoted up-regulation of the B₁ receptor. Nuclear factor-kappa B (NF- κ B) activation is very important in the synergistic upregulation of B₁ receptors, but not in B₁ agonist or IL-1 β independent up-regulation.³⁹

Regarding the early phase of the mouse pleurisy model, a significant decrease in exudate formation was observed in rats with a congenital deficiency in plasma kininogen.^{40,41} B₂ receptor antagonists reduce the exudation rate and accumulation of pleural fluid in pleurisy induced by carrageenan, and these effects could be due to the inhibition of BK capacity to up-regulate the production of cytokines such as IL-1 and IL-6.⁴²⁻⁴⁴ Thus, the B₂ receptor seems to have an important role in increased fluid leakage in the early phase of pleurisy induced by carrageenan. Neutrophil migration is absent in pleurisy induced by carrageenan in mice that lack the B₁ receptor, whereas fluid leakage formation and mononuclear

cell infiltration are similar to those observed in wild-type mice, showing that in this model neutrophil recruitment is mediated mainly by B₁ receptors, while monocyte migration and fluid formation are not.⁴⁵ These data about bradykinin receptors in pleurisy, when compared with the profile obtained in the reversal of PK11195 action by D,L-aminoglutethimide in the present study, suggest that there is a correlation between effects of the PBR ligand mediated by glucocorticoids and actions regulated by the B₂ receptor, but not those regulated by the B₁ receptor.

It has been reported that BK stimulates cytokine gene expression through activation of the transcription factor NF- κ B via the B₂ receptor and that BK-induced cytokine production is inhibited by corticosteroids.⁴⁶⁻⁴⁸ Thus, it might be supposed that PK11195 would inhibit fluid leakage and mononuclear cell migration in pleurisy via stimulation of steroid synthesis that, in turn, inhibits cytokine production through inactivation of NF- κ B. As the inhibitory action of PBR ligands on neutrophils is not via glucocorticoids, we can suppose that a PBR ligand might have the capacity to inhibit the neutrophil accumulation by a distinct mechanism that does not involve inhibition of cytokines such as IL-1. For example, the upregulation of the mechanism mediated by B₁ receptor-induced leukocyte trafficking that involves both C fibres and mast cells, and that probably occurs via substance P and histamine, is known to involve the specific up-regulation and rapid mobilisation of certain adhesion molecules for neutrophils.³⁸ In addition, B₁ agonist-promoted up-regulation of the B₁ receptor as described by Phagoo et al.³⁹ could also be implicated.

Activation of B₂ receptors in the murine model of pleurisy concerns almost exclusively the migration of mononuclear cells that is indirectly mediated by the release of several inflammatory mediators (i.e. histamine, NO and tachykinins), in addition to products derived from the arachidonic acid pathway.⁴⁹ Many of these inflammatory mediators exert their effect partly by enhancing the release of pro-inflammatory cytokines.^{42,50-52} PBR ligands reduce the macro-

phage secretion of IL-1, IL-6 and tumour necrosis factor- α .⁵³ In the pleurisy model, PK11195 caused a marked inhibition of IL-6 and IL-13 in pleural exudation. These data, together with the demonstration here that the inhibitory action of PK11195 on mononuclear cell influx in the late phase of pleurisy is dependent on glucocorticoids, suggest that this action may be partly linked to the inhibition of the release of pro-inflammatory cytokines.

PK11195 is not implicated in the modulation of this fluid formation in the late phase of pleurisy. There is little evidence for the participation of inflammatory mediators in fluid formation in this phase but prostaglandin I₂ seems to have an essential role. High quantities of cyclooxygenase-2 are expressed in mesothelial cells in the late phase of pleurisy induced by carrageenan, and this is not affected by dexamethasone.⁵⁴ The cyclooxygenase-2 expressed in mesothelial cells may be the main factor responsible for the formation of prostaglandin I₂.⁵⁵ Thus, the inability of glucocorticoids to inhibit cyclooxygenase-2 expression in mesothelial cells could explain the incapacity of PK11195 to decrease fluid leakage formation in the late phase of pleurisy.

It was previously shown that the levels of nitrate/nitrite are significantly elevated in both phases of pleurisy while only in the early phase is NO production inhibited by dexamethasone and a B₂ receptor antagonist.³⁰ Inhibitors of NOS activity reduce the development of carrageenan-induced inflammation and support a role for NO in the pathophysiology associated with this model of inflammation.^{56–59}

Dexamethasone inhibits iNOS at the transcriptional level.⁶⁰ Estrogens inhibit the increase of iNOS activity in carrageenan-induced pleurisy.⁶¹ We have now showed that PK11195 significantly inhibits nitrate/nitrite levels in the early phase of the pleurisy model and that this action is mainly regulated by glucocorticoids synthesis. Levels of nitrite/nitrate were unaffected by PK11195 in the later phase.

Thus, the hypothesis that the anti-inflammatory actions of PK11195 are correlated with its capacity to inhibit NO levels can be proposed. As this effect was reversed by D,L-aminoglutethimide, the PK11195-induced decrease in NO levels could be due mainly to the inhibition of iNOS (results not shown).

Rapid regulation of constitutive nitric oxide synthase (cNOS) activity is exerted by estrogens and glucocorticoids through activation of mitogen-activated protein kinase and PI3K/Akt pathways.^{62,63}

NO produced by endothelial nitric oxide synthase (eNOS) has previously been shown to be involved in various pathophysiological conditions, most notably in the early phase of reperfusion of various organs previously subjected to ischaemia.^{64,65} The inability of PK11195 to inhibit the exudation in the late (48 h) phase of pleurisy suggest that cNOS could have an important role in this phase.

Previous studies have shown that adenosine mediates the anti-inflammatory effects of other potent and widely used anti-inflammatory agents such as methotrexate, sulfasalazine, aspirin and sodium salicylate, but not glucocorticoids.⁶⁶ ADA, a marker of activated mononuclear cells, is an enzyme that controls the concentration of adenosine in organs and cells. Several studies have shown that it is able to suppress the effects of the released adenosine.²⁵ In the present work, pre-treatment of the animals with PK11195 caused a reduction in the mononuclear cell pool in association with a marked reduction of ADA activity in the late phase of the inflammatory response induced by carrageenan. Due to the fact that adenosine mediates the anti-inflammatory effects of PK11195 and glucocorticoids effects are not dependent on adenosine, these data suggest a complementary mechanism of action of PK11195.

In summary, the present work presents evidence that the anti-inflammatory action of PK11195 occurs in part via glucocorticoids, but an adenosine-dependent effect seems to be also implicated. We propose that glucocorticoid synthesis might cause a decrease in the actions of inflammatory mediators such as BK due in part to inhibition of the release of pro-inflammatory cytokines, but the validity of this hypothetical mechanism needs to be confirmed. Thus, this PBR ligand might be of pharmacological interest as a potential anti-inflammatory agent based on its capacity to act through several different mechanisms.

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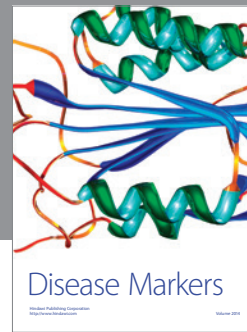
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