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Propentofylline inhibits polymorphonuclear leukocyte recruitment in vivo by a mechanism involving adenosine A_{2A} receptors

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Abstract

Propentofylline is an atypical xanthine derivative that blocks adenosine uptake and has been shown to protect against ischemia-induced cerebral damage. We have studied the effect of propentofylline on recruitment of polymorphonuclear leukocytes during acute peritonitis induced by zymosan in mice. Following i.p. injection of zymosan, recruitment of polymorphonuclear leukocytes, reflected by myeloperoxidase activity in the peritoneal cavity, increased from 2 h onwards, peaked at 4 h and then decreased gradually. Propentofylline antagonized the zymosan-induced peritoneal myeloperoxidase accumulation in a concentration-dependent manner. This effect of propentofylline was counteracted by the non-selective adenosine receptor antagonist theophylline (50 mg/kg), and by the selective adenosine A_{2A} receptor antagonists, 4-amino-8-chloro-1-phenyl-[1,2,4]-triazolo[4,3-*a*]quinoxaline (CP 66713) and 1,3-dipropyl-8-[3,4-dimethoxystyryl]-7-methylxanthine (KF 17387) (both at 2 mg/kg). The results indicate that propentofylline can reduce polymorphonuclear leukocyte recruitment in vivo and that this effect is related to an action on adenosine A_{2A} receptors.

Keywords: Propentofylline; Adenosine receptor; Xanthine; Myeloperoxidase; Polymorphonuclear leukocyte influx

1. Introduction

Polymorphonuclear leukocytes provide a primary line of defense against microbial infection, but they are also implicated in the reactions leading to cell and tissue damage after ischemia (Kochanek and Hallenbeck, 1992). Ischemia is also known to increase the levels of adenosine (Winn et al., 1981; Rudolphi et al., 1992). Adenosine is an important modulator of polymorphonuclear leukocyte function (Cronstein, 1994), and has been shown to reduce leukocyte adhesion to the endothelium (Nolte et al., 1991, 1992), reduce the expression of integrins on the polymorphonuclear leukocyte surface (Wollner et al., 1993) and reduce the release of free radicals and elastase (Cronstein et al., 1986; Zhang and Fredholm, 1994). The ischemic endothelium has been shown to release sufficient quantities of adenosine to influence polymorphonuclear leukocyte activation (Engler, 1987; Gunther and Herring, 1991).

Propentofylline (1-[5′-oxohexyl]-3-methyl-7-propylxanthine) is a xanthine derivative, that is known to decrease ischemic damage in heart (Fiedler and Komarek, 1981) and brain (DeLeo et al., 1988; Dux et al., 1990; Rudolphi et al., 1992; Parkinson et al., 1994). It is not completely understood how propentofylline produces its beneficial effects. Initial studies suggested that the effect could be due to increased cerebral blood flow (Hudlicka et al., 1981; Grome et al., 1985; Grome and Stefanovich, 1986). More recently other mechanisms have been emphasized (Rudolphi et al., 1992; Parkinson et al., 1994). In particular, it has been shown that propentofylline is able to enhance the actions of adenosine by preventing its removal by uptake (Fredholm and Lindström, 1986; Andiné et al., 1990; Fredholm et al., 1992), and to inhibit the respiratory burst of peritoneal macrophages and microglia (Banati et al., 1994). We recently found that propentofylline also inhibits the oxidative burst in polymorphonuclear leukocytes and that this effect is related to activation of adenosine receptors (Zhang and Fredholm, 1994).

Several recent studies have implicated adenosine in the mechanism of action of anti-inflammatory drugs (see Cronstein, 1994, 1995). Thus, there is evidence that two

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structurally different anti-inflammatory agents, methotrexate (Cronstein et al., 1993) and nimeluside (Capecchi et al., 1993) may reduce polymorphonuclear leukocyte activation in inflammation by increasing adenosine. In this study we therefore examined whether propentofylline was able to influence polymorphonuclear leukocytes in vivo and if adenosine was involved in these actions. As a model we used zymosan injection into the mouse peritoneum, which is known to produce a marked inflammatory response leading to activation and migration of polymorphonuclear leukocytes (Doherty et al., 1985; Lefkowith, 1988; Rao et al., 1994).

2. Materials and methods

2.1. Animals and chemicals

Male mice (BKI: NMRI) from B & K Universal (Sollentuna, Sweden) were used. They were housed ten to a cage under controlled light-dark conditions. The experiments were approved by the regional animal experimentation ethics board.

Zymosan, 3,3′,5,5′-tetramethylbenzidine (TMB), myeloperoxidase, hexadecyltri-methylammonium-bromide (HTAB), catalase, and theophylline were bought from Sigma (St. Louis, MO, USA). Propentofylline was a gift from Hoechst AG (Frankfurt am Main, Germany). 4-Amino-8-chloro-1-phenyl-[1,2,4]-triazolo[4,3-*a*]quinoxaline (CP 66713) was a gift from Dr Reinhard Sarges (Pfizer, Croton, CT, USA). 1,3-Dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KF 17387) was a gift from Dr Fumio Suzuki (Kyowa Hakko Company, Japan). TMB, CP 66713 and KF 17387 were dissolved in dimethylsulphoxide (DMSO, final concentration 0.2%). The other chemicals were dissolved in phosphate-buffered saline (PBS).

2.2. Induction of acute inflammation

Acute inflammation was induced as described by Lefkowith (1988). Mice received i.p. injections of 1 mg of zymosan in 1 ml of sterile PBS. At indicated time points after injection, mice were killed with ether anesthesia and the peritoneum was washed with 2 ml of ice-cold PBS. The abdomen was massaged for a few seconds and the fluid was withdrawn. Twenty microliters of fluid were stained for polymorphonuclear leukocytes and mononuclear cell counts. The remaining fluid was centrifuged for 5 min at $350 \times g$ at 4°C. The supernatant was decanted and the pellet was suspended in 0.5 ml of 0.05 M potassium phosphate buffer pH 6.0 containing 0.5% HTAB for myeloperoxidase assay. All samples were stored at −70°C before analysis. Samples visibly contaminated with blood were discarded. In the control group, mice received an i.p. injection of a mixture of 1 mg zymosan and indicated concentrations of propentofylline. In addition, some mice received i.p. injections of mixtures of indicated concentrations of propentofylline, zymosan and adenosine receptor antagonists (theophylline, CP 66713 or KF 17387).

2.3. Measurement of myeloperoxidase

It has been shown that the enzyme myeloperoxidase is abundant in polymorphonuclear leukocytes (Weiss, 1989) and that it is a reliable marker for the detection of polymorphonuclear leukocyte accumulation (Lundberg and Arfors, 1983). The H_2O_2-dependent oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) is catalyzed by myeloperoxidase and leads to the formation of a blue reaction product measured at 650 nm. Briefly, 200 μl of H_2O_2 was added to each well in a 96 well microtiter plate which contained 25 μl of sample or standard and 25 μl of TMB and incubated for 5 min at room temperature. The reaction was stopped by addition of 25 μl of catalase and then absorbance was read at 650 nM at 25°C with spectrophotometer (Titertek multiskan, Flow Laboratories, Finland). Values are expressed as per cent of myeloperoxidase accumulation in controls or U/ml peritoneal fluid.

2.4. Circulating and peritoneal fluid leukocyte counts

Twenty microliters of blood were taken from the orbital plexus before and at given time points after injection. Differential leukocyte counts in blood and fluids were carried out using a Bürker chamber after staining with Türk's solution. Cell numbers are expressed as $\times 10^9$/l.

2.5. Statistical analysis

All results are given as means ± standard errors of four to eight animals investigated per experimental group. Results were compared by one way analysis of variance (ANOVA) followed by Dunnett's test or by Student's *t*-test using the Primer software on a Macintosh computer. Differences were considered significant at $P < 0.05$.

3. Results

Zymosan, a particulate material obtained from yeast cell walls, was injected i.p. in mice. After 4 h, myeloperoxidase activity in peritoneal fluid, and leukocyte counts in peritoneal fluid and blood were analyzed. As shown in Table 1, zymosan (1 mg/ml) markedly increased peritoneal myeloperoxidase accumulation, indicating polymorphonuclear leukocyte influx, but decreased the number of mononuclear cells in blood.

The time course of zymosan action is shown in Fig. 1a. There was a significant increase in peritoneal myeloperoxidase accumulation from 2 h onward, which peaked at 4 h and then decreased gradually. When propentofylline (30 mg/kg) was given together with zymosan, peritoneal

Table 1
The effect of zymosan (1 mg) on myeloperoxidase accumulation (MPO; in U/ml) and leukocyte counts ($\times 10^9$/l) in peritoneal fluid (PF) and in blood of mice 4 h after i.p. injection of stimuli

	Control	Zymosan
Peritoneal MPO	0.15 ± 0.02	2.32 ± 0.12 [b]
Leukocytes in PF		
Polymorphonuclear	0.90 ± 0.12	8.50 ± 1.09 [b]
Mononuclear	1.79 ± 0.06	1.53 ± 0.12
Leukocytes in blood		
Polymorphonuclear	0.86 ± 0.26	0.93 ± 0.11
Mononuclear	5.80 ± 1.08	2.77 ± 0.43 [a]

Results are means ± standard errors of four mice in each group. [a] $P < 0.05$, [b] $P < 0.001$ (ANOVA and Dunnett's test).

myeloperoxidase accumulation at 2 h after treatment was almost abolished and the peak increase was also significantly reduced at 4 h (Fig. 1a).

The reduction in recruitment of polymorphonuclear leukocytes could not be accounted for by a decreased

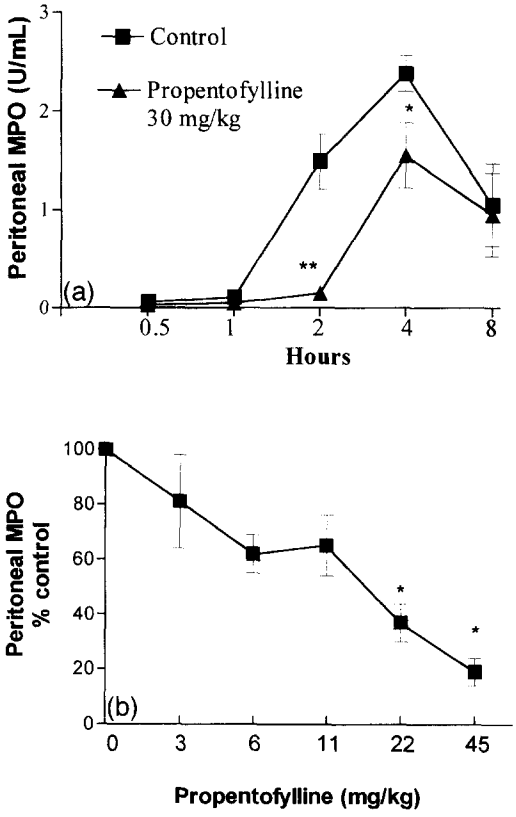

Fig. 1. Antagonism by propentofylline of zymosan (1 mg)-induced myeloperoxidase accumulation. *Panel a.* Effects in the absence or presence of propentofylline (30 mg/kg). *Panel b.* Dose-related inhibition by propentofylline of peritoneal myeloperoxidase accumulation 2 h after injection of 1 mg of zymosan. Results represent mean and standard errors of four to eight animals in each group. Results are U/ml or normalized to percentage of control values. * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test or ANOVA and Dunnett's test).

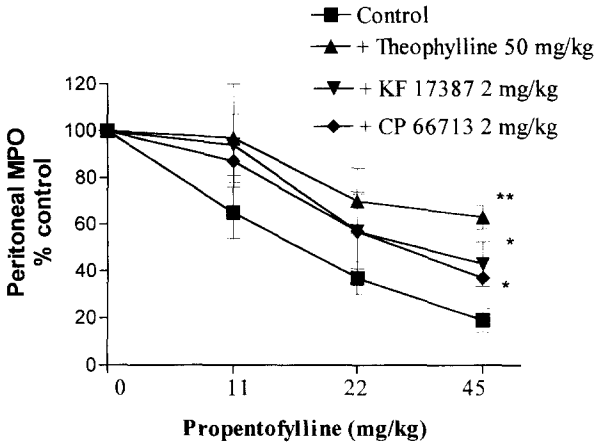

Fig. 2. Peritoneal myeloperoxidase accumulation after i.p. injection in mice of the adenosine antagonists theophylline (50 mg/kg), KF 17387 (2 mg/kg), or CP 66713 (2 mg/kg), together with propentofylline (30 mg/kg) and zymosan (1 mg). The mice were killed after 2 h. Mean and standard errors of four to seven in each group. Results are normalized to per cent of control values (which were 0.52 ± 0.15, 0.42 ± 0.06, 0.65 ± 0.05 and 0.65 ± 0.12 U/ml in control, theophylline, KF 17387 and CP 66713 groups, respectively). * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

number of these cells in blood. At 1 and 2 h after zymosan, the blood counts were significantly higher in the propentofylline-treated group (2.5 ± 0.22 and 3.90 ± 0.56 $\times 10^9$ cells per liter in the group treated with propentofylline vs. 1.28 ± 0.26 and 1.41 ± 0.44 $\times 10^9$ cells per liter in the untreated group).

Based on the time course we chose the 2 h time point to examine the effect of different doses of propentofylline on the acute inflammation response. Propentofylline caused a clear, dose-dependent (from 3 to 45 mg/kg) inhibition of zymosan-induced peritoneal myeloperoxidase accumulation (Fig. 1b). The maximal inhibition was 81%.

We next examined if the effects of propentofylline were blocked by adenosine receptor antagonists. Theophylline (50 mg/kg), a non-selective adenosine A_1 and A_2 receptor antagonist (Fredholm et al., 1994), blocked the inhibitory effects of propentofylline on peritoneal myeloperoxidase accumulation (Fig. 2). The antagonism was almost complete against the lowest dose of propentofylline (11 mg/kg) and about 60% at the highest dose (45 mg/kg). Theophylline given alone tended to inhibit peritoneal myeloperoxidase accumulation, but this was not statistically significant.

In order to determine the type of adenosine receptor involved, we tested the effects of KF 17387 and CP 66713, which are selective adenosine A_{2A} receptor antagonists (see Fredholm et al., 1994). Both KF 17387 (2 mg/kg) and CP 66713 (2 mg/kg) reduced the effects of propentofylline (Fig. 2). Both drugs are poorly water soluble and had to be dissolved in 0.2% DMSO. No significant inhibitory effects of DMSO on myeloperoxidase accumulation were seen in a separate group of four mice (data not

shown). However, the poor water solubility prevented testing of higher doses of the adenosine A_{2A} receptor antagonists.

4. Discussion

The objective of this study was to examine if propentofylline can influence inflammatory activation of leukocytes in vivo. For this purpose we used a previously described model for zymosan-induced peritoneal inflammation in the mouse which provides a means for reproducible induction of pronounced peritoneal polymorphonuclear leukocyte accumulation (Doherty et al., 1985; Lefkowith, 1988; Rao et al., 1994). As studied in mice and rats, the peritoneal response to zymosan has been found to be a complex inflammatory reaction involving activation of both mast cells and macrophages, and leading to release of mediators such as leukotrienes, prostaglandins, histamine, interleukin-1, tumor necrosis factor, and platelet-activating factor (Doherty et al., 1985; Lefkowith, 1988; Tarayre et al., 1989; Perretti et al., 1992; Damas and Prunescu, 1993; Rao et al., 1994; Nathens et al., 1995; Tordjman et al., 1995). Zymosan-induced peritoneal polymorphonuclear leukocyte recruitment has been shown to be reduced by a range of antiinflammatory agents, including glucocorticoids, colchicine, 5-lipoxygenase inhibitors, and antibodies to interleukin-1 and leukocytic β_2-integrins (Lefkowith, 1988; Griffiths et al., 1991; Rao et al., 1994; Van de Langerijt et al., 1994).

In line with previous observations (Lefkowith, 1988), very few mononuclear leukocytes were recruited to the peritoneal cavity during the 4 h incubation with zymosan. Nonetheless, zymosan caused a significant reduction in the number of circulating mononuclear leukocytes, a well known phenomenon in many types of inflammation, that is believed to result from redistribution of lymphocytes from the blood to lymphoid tissues (e.g. Remick et al., 1990; Van der Poll et al., 1992; Hawes et al., 1993).

A major finding of the present study is that also propentofylline is powerful inhibitor of zymosan-induced polymorphonuclear leukocyte recruitment. Moreover, the effect probably involves adenosine acting at adenosine A_{2A} receptors since the effect of propentofylline was blocked not only by non-selective but also by adenosine A_{2A} receptor selective antagonists. Both CP 66713 (Sarges et al., 1990) and KF 17387 (Shimada et al., 1992) are potent and selective adenosine A_{2A} receptor antagonists. The fact that two structurally different compounds (KF 17387 is a xanthine; CP 66713 is not) antagonized propentofylline provides good evidence that its effects are due to activity at adenosine A_{2A} receptors. The non-selective antagonist theophylline per se slightly inhibited zymosan-induced peritoneal myeloperoxidase accumulation. The mechanism responsible for this possible effect was not examined, but it is well known that theophylline can act as cyclic AMP phosphodiesterase inhibitor. In this respect it is not much more potent than propentofylline (Fredholm and Lindgren, 1984). However, the fact that the two compounds generally had opposite effects obviously cannot be explained by postulating that they have a similar mechanism of action.

As noted in the Introduction there is good evidence that propentofylline is able to enhance the effects of adenosine by blocking its cellular uptake (Fredholm et al., 1992; Fredholm and Lindström, 1986; Parkinson et al., 1994). Indeed, an inhibition of adenosine inactivation appears to explain the actions of propentofylline on human polymorphonuclear leukocytes in vitro (Zhang and Fredholm, 1994). It has also been shown that NBTI (*S*-(*p*-nitrobenzyl)-6-thioinosine), a known adenosine uptake inhibitor, decreases leukocyte-endothelium interaction by an action involving endogenous adenosine (Nolte et al., 1992).

The inhibitory effect of propentofylline on zymosan-induced myeloperoxidase activity in the peritoneum cannot be explained by neutropenia, since the number of circulating polymorphonuclear leukocytes was, if anything, increased by the drug. It is similarly unlikely that it is due to a major circulatory derangement. Thus, administration of 30 mg/kg propentofylline intraduodenally or by continuous intravenous infusion to dogs causes only minor decreases in arterial blood pressure (Hoechst AG, data on file). After a single i.v. bolus injection of propentofylline there is a slight reduction in blood pressure and a rise in heart rate, but this is over within minutes (data on file). Thus, unless mice are very different from dogs with respect to the effects of propentofylline on the circulation, any minor circulatory effect of the doses given would be too transitory to account for the actions reported here.

Adenosine A_1 receptor agonists are better inhibitors of pleural and peritoneal inflammation in vivo than adenosine A_2 receptor agonists (Schrier et al., 1990; Lesch et al., 1991). On the other hand, it has been shown that a selective adenosine A_{2A} receptor agonist CGS 21680 (see Fredholm et al., 1994) reduces postischemic leukocyte-endothelium interaction, whereas 2-chloro-N^6-cyclopentyladenosine, an adenosine A_1 receptor agonist is ineffective (Nolte et al., 1992; Cronstein et al., 1992). Furthermore, the inhibitory effect of adenosine analogs on polymorphonuclear leukocyte oxidative burst and release of enzymes is due to an activation of adenosine A_{2A} receptors (Fredholm et al., 1996). Thus, the effect of propentofylline, which appears to be related to adenosine A_{2A} receptors, may be due to an inhibition of the actions of polymorphonuclear leukocytes rather than of the release of inflammatory mediators, but the design of the study does not permit further conclusions about the process(es) affected by propentofylline.

In summary, we provide evidence that propentofylline depresses the polymorphonuclear leukocyte influx to an inflamed site. Based on the present and previous data we postulate that propentofylline, as an inhibitor of adenosine

uptake, increases the actions of endogenous adenosine. Our data are in line with recent reports that drugs that increase adenosine levels may act as antiinflammatory agents (see Cronstein, 1994, 1995). Given the role of polymorphonuclear leukocytes in producing ischemia-related tissue damage it is an intriguing possibility that also the established neuroprotective effect of propentofylline might in part be mediated by reduced activation of polymorphonuclear leukocytes.

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