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Fenspiride: an anti-inflammatory drug with potential benefits in the treatment of endotoxemia

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Abstract

Using a model of endotoxemia triggered by the intravenous injection of bacterial lipopolysaccharide (0.1 and 1 mg/kg) to guinea-pigs, we investigated the interference of fenspiride, an anti-inflammatory drug recommended for the treatment of inflammatory diseases of the upper respiratory tract. Administered orally at 60 mg/kg, fenspiride reduced the lipopolysaccharide-induced early rise of tumor necrosis factor concentrations in serum (4.2 ± 0.9 vs. 2.3 ± 0.5 ng/ml, $P < 0.05$) and in the bronchoalveolar lavage fluid (55.7 ± 20 vs. 19.7 ± 7.5 ng/ml, $P < 0.05$). The lipopolysaccharide-induced primed stimulation of alveolar macrophages, defined as their enhanced release of arachidonic acid metabolites as compared to cells from untreated controls upon stimulation with *N*-formyl-methionyl-phenylalanine was also reduced by fenspiride (1551.5 ± 183.7 vs. 771.5 ± 237.5 pg/μg protein, $P < 0.05$ for thromboxane B_2 and 12.6 ± 4.9 vs. 3.6 ± 0.9 pg/μg protein, $P < 0.05$ for leukotriene C_4). Finally, fenspiride reduced the increased serum concentrations of extracellular type II phospholipase A_2 (3.9 ± 1.2 vs. 1.2 ± 0.1 nmol/ml per min, $P < 0.01$), the intensity of the neutrophilic alveolar invasion and the lethality due to the lipopolysaccharide. The protective effect of fenspiride may result from the inhibition of the formation of tumor necrosis factor, a major mediator of the effects of lipopolysaccharide.

Keywords: Fenspiride; Lipopolysaccharide; TNF (tumor necrosis factor); Alveolar macrophage, guinea-pig; Phospholipase A_2

1. Introduction

Recent evidence suggests that fenspiride, an anti-inflammatory drug with anti-bronchoconstrictor properties (Advenier, 1988), inhibits the release of tumor necrosis factor α (TNFα) (Cunha et al., 1993), a cytokine mediator released during lipopolysaccharide-induced shock.

The injection of lipopolysaccharide to experimental animals causes a massive lung infiltration of neutrophils and the priming or the activation of macrophages, leading to an increased release of arachidonic acid metabolites and of proinflammatory cytokines, which all contribute to the pathogenesis of septic shock (Morrison and Ryan, 1987). Recent studies on the host response to endotoxins have also stressed the importance of the increased activity of the lipolytic enzyme phospholipase A_2, measured in serum of endotoxemic animals or patients with septic shock (Vadas and Hay, 1983).

Phospholipases A_2, which hydrolize the sn-2 fatty acid acyl ester bond of phosphoglycerides, exist in different forms that vary in molecular weight, pH optima, Ca^{2+} dependence and substrate specificity (Vadas et al., 1993; Mayer and Marshall, 1993). A high molecular weight phospholipase A_2 (about 80 kDa) has been characterized in many tissues and cells, including macrophages (Goppelt-Struebe and Rehfeldt, 1992). This enzyme functions intracellularly and preferentially cleaves arachidonic acid or other polyunsaturated fatty acids, suggesting a role in eicosanoid biosynthesis. In addition, a 14 kDa molecular weight phospholipase A_2, similar to that found in snake venoms, has been described in inflammation. This enzyme, often referred as type II non-pancreatic mammalian phospholipase A_2, functions both as cell-associated or extracellularly

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and has little preference for a particular fatty acid in the sn-2-acyl position (Vadas et al., 1993; Mayer and Marshall, 1993).

Macrophages can be activated or primed by lipopolysaccharide for an enhanced release of arachidonic acid metabolites in response to a second stimulus, reflecting a modification of the activity of the intracellular high molecular weight phospholipase A_2. An in vivo example of this phospholipase A_2 modification occurs in the guinea-pig upon the intravenous injection of lipopolysaccharide, since alveolar macrophages subsequently stimulated in vitro with the chemotactic peptide *N*-formyl-methionyl-phenylalanine (fMLP) release increased amounts of arachidonic acid metabolites as compared to cells from untreated controls. In the same model, the intravenous administration of lipopolysaccharide induces a rise in circulating phospholipase A_2, an enzyme recognized in rabbits as the distinct extracellular form assigned to the type II mammalian phospholipase A_2 (Vadas et al. 1993).

We now assessed the capacity of fenspiride to interfere with some aspects of endotoxic shock in the guinea-pig, particularly the potentiation of alveolar macrophages for an increased capacity to release arachidonic acid metabolites as a consequence of the activation of the high molecular weight phospholipase A_2, the rise in circulating concentrations of type II low molecular weight phospholipase A_2, the neutrophil lung recruitment and the increased concentration of TNF. We provide evidence for an in vivo protective effect of fenspiride in the course of experimental endotoxemia.

2. Materials and methods

2.1. Animals and treatments

Male Dunkin-Hartley guinea-pigs weighing 400–600 g were purchased from Lebau, Gambais, France. The animals had free access to food (U.A.R., Epinay-sur-Orge, France) and water and were maintained for a maximum of 2 weeks before the experiments. They were divided into four groups: saline (control), lipopolysaccharide alone, fenspiride alone and lipopolysaccharide-fenspiride. Lipopolysaccharide was injected into the saphenous vein (0.1–1 mg/kg, from *Escherichia coli* 055:B5, Difco, Detroit, USA) dissolved in 0.9% NaCl 1 h, 6 h or 24 h before the experiments. Fenspiride, [8-(2-phenylethyl)-1-oxa-3,8-diazaspiro(4, 5)decan-2-one] chlorhydrate (Servier, Courbevoie, France) was dissolved in 0.9% NaCl and given orally at 20–60 mg/kg once a day during 3 days and 1 h before the lipopolysaccharide injection. In some experiments, a single dose of fenspiride was administered orally (60 mg/kg), at the time of the lipopolysaccharide administration. Saline (control) animals were treated similarly with 0.9% Na Cl.

2.2. Isolation and stimulation of alveolar macrophages

Alveolar macrophages were recovered by bronchoalveolar lavages performed in anesthetized guinea-pigs (sodium pentobarbital, 40 mg/kg, i.p., Sanofi, Libourne, France) through a plastic cannula inserted into the trachea. First, 5 ml of 0.9% NaCl solution were injected and reaspirated at room temperature. Then, 10 ml of 0.9% NaCl were flushed back and forth 5 times before being collected and pooled with the first lavage fluid (the final volume was 11–13 ml). Finally, lungs were further lavaged with 10×5 ml NaCl 0.9% and the fluid was recovered in a separate vial. Bronchoalveolar lavages were centrifuged ($470 \times g$, 10 min, room temperature) and the cell-free supernatant from the first sample (11–13 ml) was stored at −20°C for soluble phospholipase A_2 and TNF determination. This lavage procedure was designed to prevent dilution of soluble phospholipase A_2 or TNF recovered in a high volume of bronchoalveolar lavage required to obtain an appropriate number of alveolar cells. The cells obtained from the two pellets were pooled and suspended at 10^6 cells/ml in RPMI 1640 medium (Gibco, Paisley, Scotland), supplemented with 3% fetal calf serum and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, Gibco, Paisley, Scotland). Two ml of this suspension were plated in 35 mm diameter tissue culture wells (6-well, Falcon, Becton Dickinson, New Jersey, USA) and cells were allowed to adhere in a CO_2 incubator at 37°C. After 1 h, non adherent cells were removed by rinsing the dishes with RPMI medium. The remaining adherent cells consisted in > 95% alveolar macrophages as evaluated microscopically after staining the monolayers with the Diff-Quik set (Baxter Dade AG, Düdingen, Switzerland). The adherent cells were covered with 1 ml serum-free RPMI medium and further incubated for 1 h. The stimulus fMLP (Sigma, St. Louis, USA, 10^{-2} M stock solution prepared in dimethyl sulfoxide) was added at this point in increasing concentrations (1 nM to 1 μM) in RPMI medium and the incubation was continued for 10 min. Supernatants were kept at −20°C for thromboxane B_2 and leukotriene C_4 determinations.

2.3. Determination of thromboxane B_2 and leukotriene C_4

Thromboxane A_2 was determined as its stable metabolite thromboxane B_2 by radioimmunoassay (Sors et al., 1978). Briefly, samples (100 μl) were incubated overnight at 4°C with fixed amounts of antibody and [^{125}I] thromboxane B_2 (L.P.R.I. Institut Pasteur, Paris, France). The bound antibody-thromboxane B_2 complex

was precipitated by polyethylenglycol 6000 (30%, Merck, Darmstadt, Germany) and the radioactivity of the pellet was counted. The monoclonal antibody employed was less than 0.02% crossreactive with prostaglandins E_2, D_2, $F_{2\alpha}$, 6-keto$F_{1\alpha}$ and arachidonic acid. The sensitivity of the assay was approximately 2 pg/100 μl.

For the leukotriene C_4 radioimmunoassay, samples (100 μl) were incubated overnight at 4°C with antibody (kindly provided by Drs. F. Kohen and U. Zor, Weizmann Institute, Rehovot, Israel) and 14,15(*n*)-[^{3}H]-leukotriene C_4 (Amersham, Buckinghamshire, U.K.). Dextran-coated charcoal (code C-5260, Sigma, St. Louis, USA) was used to separate unbound ligand by centrifugation and the antibody-leukotriene C_4 complex remained in the supernatants. Radioactivity was measured in 0.5 ml of supernatants after mixing with 4.5 ml of an aqueous counting scintillant (ACS, Amersham, Buckinghamshire, UK). The monoclonal anti-leukotriene C_4 antibody was 10% crossreactive with leukotriene D_4 and less than 0.1% with leukotriene A_4 and leukotriene B_4. The sensitivity of the assay was approximately 30 pg/100 μl (Aehringhaus et al., 1982).

2.4. Determination of extracellular phospholipase A_2

Extracellular phospholipase A_2 was determined fluorometrically using a phospholipid labeled at the sn-2-acyl position with 1-pyrenyldecanoic acid (Radvanyi et al., 1989). The substrate used, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-sn-glycero-3-phosphoglycerol (Molecular Probes, OR, USA) forms vesicles in the aqueous reaction medium and the fluorescence of the pyrene group is negligible due to pyrene-pyrene interaction. Following hydrolysis by phospholipase A_2, the fluorescent fatty acid liberated tightly binds to serum albumin (fatty acid-free bovine albumin, Sigma, St. Louis, USA) and the pyrene fluorescence produced can be monitored by spectrofluorometry. The fact that the monomer fluorescence of 1-pyrene phospholipids is very low in the presence of serum albumin whereas that of 1-pyrenyldecanoic acid is high and proportional to its concentration makes it possible to measure the hydrolysis of 1-pyrene phospholipids by phospholipase A_2. The fluorescent substrate was suspended in ethanol at the concentration of 0.2 mM and vesicles were prepared by mixing vigorously in a 4×10 mm plastic cuvette: 10 μl of the substrate solution (final concentration 2 μM) to 970 μl of reaction medium containing 50 mM Tris-HCl pH 9, 0.5 M NaCl and 1 mM EGTA. Following the addition of 10 μl serum albumin (final concentration 0.1%), 10 μl test enzyme and 10 μl $CaCl_2$ 1 M (final concentration 10 mM), the fluorescence was monitored in a Jobin et Yvon JY3D spectrofluorometer (Longjumeau, France) equipped with a Xenon lamp, using an excitation and an emission wavelength of 345 and 398 nm, respectively. Phospholipase A_2 was estimated according to the formula: phospholipase A_2 (μmol/min) $= 2 \times 10^{-4}$ $(S - S_0) \times V/F_{max}$ in which S = slope of the curve representing the increase of fluorescence in the presence of the test enzyme versus time (min), S_0 = slope in the absence of the test enzyme, V = volume of the substrate (μl) and F_{max} = maximal fluorescence signal corresponding to the fluorescence emission of 5 μg standard venom phospholipase A_2 (from Naja naja venom, Sigma, St. Louis, USA). The sensitivity of the assay was approximately 50 pmol of substrate hydrolyzed/min per ml enzyme sample. Assays were performed in serum and bronchoalveolar lavages. The in vitro effect of fenspiride on circulating lipopolysaccharide-induced type II phospholipase A_2 activity, was also assessed after incubation of serum with fenspiride (10 min to 6 h, 10^{-5}–10^{-3} M, 37°C).

2.5. Determination of tumor necrosis factor

TNF concentrations were measured in serum and bronchoalveolar lavages from guinea-pigs by cytotoxicity on fibrosarcoma cells (WEHI 164 clone 13 line, kindly provided by Dr. F.J. Zijlstra, Erasmus University, Rotherdam, The Netherlands). WEHI 164 cells were grown in Dulbecco's medium (Gibco, Paisley, Scotland) supplemented with 10% inactivated fetal calf serum (Boehringer, Mannheim, Germany) and antibiotics (1% w/v gentamycin, and 1% w/v amphotericin B, Boehringer, Mannheim, Germany) in an humidified atmosphere of 5% CO_2. Cells (10^6/ml) were incubated for 3 h in the presence of 1 μg/ml actinomycin D (Sigma, St. Louis, USA). Aliquots of this cells suspension (50 μl/well containing 5×10^4 cells) were plated in 96-well flat bottom microtiter plates (Nunclon Delta, Roskilde, Denmark) and incubated for 24 h with 50 μl samples or TNFα standard dilutions (10–10^4 pg/ml, human recombinant TNFα, Bender-Wien, Vienna, Austria) in triplicate. The plates were further incubated for 24 h with added 50 μl/well XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate) labeling mixture prepared as recommended by the manufacturer (Cell proliferation kit II XTT, Boehringer, Mannheim, Germany). Optical density was measured in an automatic reader with a test wavelength of 490 nm and a reference wavelength of 630 nm (Dynatech MR 5000, Marnes La Coquette, France). The identity of the effector molecule as TNFα could not be demonstrated directly since available neutralizing antibodies do not recognize guinea-pig TNFα. However, in assays repeated using supernatants from human monocytes, cytotoxic activity was completely inhibited by a polyclonal rabbit anti-human TNFα anti-serum (a gift of S. Mamas, Institut Pasteur, Paris, France).

2.6. Determination of cyclic AMP concentrations

Alveolar macrophages purified from bronchoalveolar lavages as described earlier were incubated for 3 min at 37°C with Tris-HCl buffer (50 mM, pH 7.4) alone or in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 10^{-3} M, Sigma, St. Louis, USA) or fenspiride (10^{-5}, 10^{-4}, 10^{-3} M). The reaction was stopped by heating the plates in boiling water for 3 min to recover the intracellular cyclic AMP (cAMP) content in the extracellular medium that was analyzed using the competitive protein-binding assay described by Gilman, (1970). For each experiment, a single pool of macrophages was prepared from one guinea-pig from which six dishes (35 mm diameter) were plated for different in vitro treatments. Five separate experiments were performed in normal untreated guinea-pigs.

2.7. Additional methods

Blood samples were obtained by cardiac puncture and serum prepared by centrifugation at 470 × g for 5 min, was stored at −20°C until assayed. Total numbers of leukocytes were determined in blood and bronchoalveolar lavage samples using a Coulter counter (model ZM, Coultronics, Margency, France). Differential counts were performed in blood smears or bronchoalveolar lavages cytocentrifuge preparations (Hettich Universal, Tuttlingen, Germany), after staining with the Diff-Quik stain (Baxter Dade AG, Düdingen, Switzerland). The protein content was measured in cell scrapings solubilized in 0.1% Triton X-100 (Sigma, St. Louis, USA) with the bicinchoninic acid kit purchased from Pierce (IL, USA), (Smith et al., 1985).

2.8. Data analysis

All data are presented as the mean ± standard error of the mean (S.E.M.). Comparisons between means were performed by the Mann-Whitney two sample U-test for unpaired data.

3. Results

3.1. Neutrophil infiltration into the alveolar space

Total leukocyte counts in blood samples from lipopolysaccharide-treated guinea-pigs revealed an early leukopenia (6389 ± 676 vs. 2419 ± 261 leukocytes/μl 1 h after the lipopolysaccharide challenge in control and lipopolysaccharide-treated guinea-pigs, respectively, $n = 6$, $P < 0.01$), that gradually reversed into an increased number of circulating leukocytes 24 h later (6945 ± 421 vs. 13368 ± 960 leukocytes/μl in control and lipopolysaccharide-treated guinea-pigs, respectively, $n = 6$, $P < 0.01$). Those changes were not affected by fenspiride. In comparison, non significant differences were observed in bronchoalveolar lavage leukocyte counts between the different groups.

Differential cell analysis of blood samples revealed that changes in neutrophil numbers paralleled those of total leukocyte counts and were also not affected by fenspiride. As compared to control values (1519 ± 190 neutrophils/μl, $n = 6$), lower neutrophil numbers (667 ± 192 neutrophils/μl, $n = 6$, $P < 0.05$) were observed

Table 1
Effect of fenspiride on the neutrophil recruitment induced by lipopolysaccharide (LPS) in bronchoalveolar lavages from guinea-pigs

	Time post-LPS injection		
	1 h	6 h	24 h
Control	0.36 ± 0.1	0.40 ± 0.1	0.34 ± 0.1
	2.6 ± 0.2%	2.8 ± 0.2%	2.3 ± 0.8%
	($n = 4$)	($n = 4$)	($n = 6$)
Fenspiride 20 mg/kg	0.30 ± 0.1	0.33 ± 0.1	0.41 ± 0.1
	2.4 ± 0.5%	2.5 ± 0.5%	3.0 ± 0.2%
	($n = 4$)	($n = 4$)	($n = 6$)
Fenspiride 60 mg/kg	0.32 ± 0.1	0.65 ± 0.1	0.65 ± 0.1
	2.9 ± 0.4%	6.2 ± 0.6%	5.4 ± 0.9%
	($n = 6$)	($n = 6$)	($n = 6$)
LPS 0.1 mg/kg	0.45 ± 0.1	0.53 ± 0.2	1.60 ± 0.89 [a]
	4.5 ± 0.7%	5.8 ± 1.1%	14.0 ± 7.0%
	($n = 6$)	($n = 6$)	($n = 6$)
LPS/fenspiride 20 mg/kg	0.63 ± 0.1%	0.58 ± 0.4	1.65 ± 0.5 [a]
	4.5 ± 0.3	4.3 ± 1.5%	14.8 ± 2.9
	($n = 4$)	($n = 4$)	($n = 6$)
LPS/fenspiride 60 mg/kg	0.25 ± 0.1	0.62 ± 0.2	0.69 ± 1.1 [b]
	3.6 ± 0.7%	5.5 ± 1.3%	5.0 ± 6.0%
	($n = 6$)	($n = 6$)	($n = 6$)

Results are presented as the means ± S.E.M. of the neutrophil numbers (× million) or the percent neutrophils of total leukocytes recovered. n = number of animals; [a] = $P < 0.05$ vs. control; [b] = $P < 0.05$ vs. LPS alone.

in blood from 1 h lipopolysaccharide-treated guinea-pigs and this tendency reversed after 24 h (9733 ± 636 neutrophils/μl, $n = 6$, $P < 0.05$ in lipopolysaccharide-treated guinea-pigs). In bronchoalveolar lavages, small changes of neutrophil numbers were observed between the different groups at 1 or 6 h after the lipopolysaccharide treatment. However, 24 h after the lipopolysaccharide challenge, a 5–6-fold increase in the number of neutrophils in the bronchoalveolar lavage was noted, which was significantly reduced by fenspiride (60 mg/kg) (Table 1). At the lower dose of 20 mg/kg, fenspiride did not affect significantly the number of neutrophils retrieved in the bronchoalveolar lavage (Table 1).

3.2. Release of arachidonic acid metabolites by lipopolysaccharide-primed alveolar macrophages stimulated by fMLP

Alveolar macrophages from control or lipopolysaccharide-treated guinea-pigs produced similar basal amounts of arachidonic acid metabolites. Non-stimulated alveolar macrophages released 15.0 ± 5.5 and 10.5 ± 9.5 pg thromboxane B_2/μg protein ($n = 6$) in control and 1 h lipopolysaccharide (0.1 mg/kg)-treated animals, respectively. Values for leukotriene C_4 release were 0.5 ± 0.4 and 0.6 ± 0.2 pg/μg protein ($n = 6$) in control and 1 h-lipopolysaccharide (0.1 mg/kg) treated animals, respectively. However, in response to the subsequent stimulation by 0.1 μM fMLP, cells from 1 h lipopolysaccharide-treated animals (0.1 mg/kg, i.v.) produced significantly increased amounts of thromboxane B_2 and leukotriene C_4 than did cells from untreated guinea-pigs. This process, in which circulating lipopolysaccharide potentiates the release of arachidonic acid metabolites when fMLP is used as a second stimulus, was designed as primed stimulation. When alveolar macrophages from lipopolysaccharide-treated guinea-pigs treated orally with fenspiride (60 mg/kg) were stimulated by fMLP, the production of both thromboxane B_2 or leukotriene C_4 were reduced to levels similar to those of untreated cells (Fig. 1), indicating that fenspiride inhibits the in vivo primed stimulation induced by lipopolysaccharide. This inhibition was not observed in cells from guinea-pigs pretreated with 20 mg/kg of fenspiride (Fig. 1). Fenspiride alone (20–60 mg/kg) did not induce significant changes in thromboxane B_2 or leukotriene C_4 release by either resting or stimulated macrophages.

3.3. Serum concentrations of extracellular phospholipase A_2 activity

Basal activity of phospholipase A_2 was detected in bronchoalveolar lavages and supernatants from alveolar macrophages of control guinea-pigs, which was not

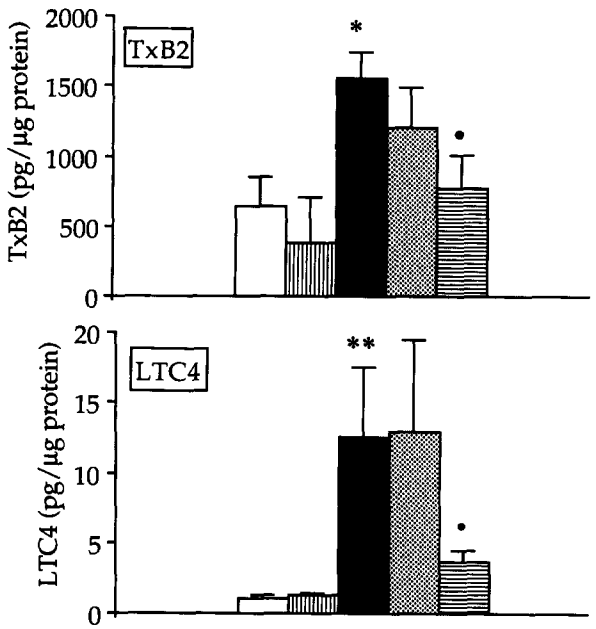

Fig. 1. Interference of fenspiride with the release of thromboxane B_2 (TxB_2) and leukotriene C_4 (LTC_4) by macrophages primed by lipopolysaccharide and stimulated by fMLP. Alveolar macrophages were isolated from control guinea-pigs (open bars), fenspiride-treated guinea-pigs (60 mg/kg per day, orally for 3 days, vertical lined bars), 1 h lipopolysaccharide-treated guinea-pigs (0.1 mg/kg i.v., dark bars), 1 h lipopolysaccharide guinea-pigs pretreated with fenspiride (20 mg/kg, dotted bars) or 1 h lipopolysaccharide guinea-pigs pretreated with fenspiride (60 mg/kg, horizontal lined bars). Cells were stimulated by fMLP (0.1 μM) for 10 min and supernatants were kept at −20°C for thromboxane B2 and leukotriene C4 radioimmunoassays. Results are presented as the mean ± S.E.M. from 6 guinea-pigs. $^{*}P < 0.05$, $^{**}P < 0.01$ vs control, $^{\bullet}P < 0.05$ vs lipopolysaccharide alone.

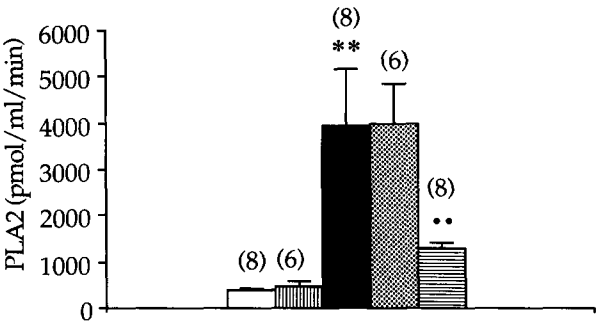

Fig. 2. Interference of fenspiride with extracellular phospholipase A_2 (PLA_2) in serum from endotoxemic guinea-pigs. Phospholipase A_2 activities were measured fluorometrically as described in Materials and methods in serum from control guinea-pigs (open bars), fenspiride-treated guinea-pigs (60 mg/kg per day, orally for 3 days, vertical lined bars), 6 h lipopolysaccharide-treated guinea-pigs (0.1 mg/kg i.v., dark bars), 6 h lipopolysaccharide guinea pigs pretreated with fenspiride (20 mg/kg, dotted bars) or 6 h lipopolysaccharide guinea-pigs pretreated with fenspiride (60 mg/kg, horizontal lined bars). Results are presented as the means ± S.E.M. from the number of guinea-pigs shown in parentheses. $^{**}P < 0.01$ vs. control, $^{\bullet\bullet}P < 0.01$ vs. lipopolysaccharide alone.

affected either by lipopolysaccharide or by fenspiride over the period of observation. However, approximately a ten fold increase in circulating phospholipase A_2 activity was observed in guinea-pigs treated for 6 h with lipopolysaccharide (0.1 mg/kg) which was significantly reduced by fenspiride (60 mg/kg, for three days before the lipopolysaccharide challenge). Pretreatment with 20 mg/kg was ineffective (Fig. 2). When a single dose of fenspiride (60 mg/kg) was administered at the time of the lipopolysaccharide challenge, phospholipase A_2 activity was slightly reduced from 3966.6 ± 1280.5 pmol/ml per min in serum from lipopolysaccharide-treated guinea-pigs (0.1 mg/kg, $n = 8$) to 2672.5 ± 572.8 in lipopolysaccharide-fenspiride guinea-pigs ($n = 6$). In addition, phospholipase A_2 activity was not affected when serum from lipopolysaccharide-treated guinea pigs was incubated in vitro with fenspiride (10 min–6 h, 1 mM) (data not shown).

3.4. Intracellular cyclic AMP concentrations in alveolar macrophages

Mean baseline concentrations of cAMP in untreated alveolar macrophages were not significantly different from those observed in cells after exposure to different doses of fenspiride (10^{-5}, 10^{-4}, 10^{-3} M, 3 min, 37°C). In comparison, a significantly higher cAMP content was measured when alveolar macrophages were exposed to IBMX (10^{-3} M, 3 min, 37°C): 5.5 ± 0.2 pmol/mg protein in control conditions, 9.5 ± 0.15 pmol/mg protein in macrophages exposed to fenspiride (10^{-3} M) and 26.5 ± 7.5 pmol/mg protein in macrophages exposed to IBMX 10^{-3} M. Values represent means ± S.E.M. from five separate experiments.

3.5. Tumor necrosis factor concentrations in serum and in bronchoalveolar lavages

The injection of lipopolysaccharide (0.1 mg/kg) induced a marked rise of TNF concentrations in both serum and bronchoalveolar lavages that peaked at 1 h after the lipopolysaccharide challenge and declined thereafter. The lipopolysaccharide-increased concentrations were significantly reduced in guinea-pigs pretreated with the high dose of fenspiride (60 mg/kg) but remained unchanged after treatment with the low dose (20 mg/kg, Fig. 3). A single dose of fenspiride (60 mg/kg), administered at the time of the lipopolysaccharide challenge, slightly reduced TNF concentrations: 4234 ± 993 vs. 3812 ± 227 pg/ml ($n = 6$) in serum from lipopolysaccharide alone and lipopolysaccharide-fenspiride treated guinea-pigs, respectively and 55673 ± 20005 vs. 35504 ± 3697 pg/ml ($n = 6$) in bronchoalveolar lavages from lipopolysaccharide alone and lipopolysaccharide-fenspiride guinea-pigs, respectively. Basal concentrations of TNF in serum and bronchoalveolar lavages were very low (12.0 ± 3.1 and 14.4 ± 2.6 pg/ml in serum and bronchoalveolar lavages, respectively) and were not affected by fenspiride.

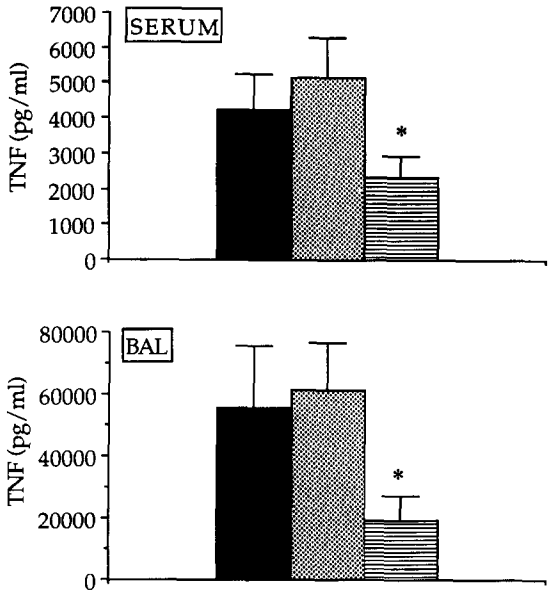

Fig. 3. Interference of fenspiride with TNF concentrations of serum and bronchoalveolar lavages (BAL) from endotoxemic guinea-pigs. TNF activity was measured by cytotoxicity on WEHI 164 cells as described in Materials and methods in serum and bronchoalveolar lavages from guinea-pigs treated for 1 h with lipopolysaccharide (0.1 mg/kg i.v., dark bars), 1 h lipopolysaccharide guinea-pigs pretreated with fenspiride (20 mg/kg per day, orally, for 3 days, dotted bars) or 1h lipopolysaccharide guinea-pigs pretreated with fenspiride (60 mg/kg per day, orally, for 3 days, vertical lined bars). Values from control or guinea-pigs treated with fenspiride alone are very low (< 20 pg/ml) and do not appear in the graph. Results are presented as the means ± S.E.M. from six guinea-pigs. $^*P < 0.05$ vs. lipopolysaccharide alone.

3.6. Studies on lethality

Intravenous injection of 1 mg/kg of lipopolysaccharide to guinea-pigs resulted in a 50% and 83% mortality at 24 and 72 h, respectively ($n = 12$). In guinea-pigs daily treated with fenspiride (60 mg/kg for 3 days) before the lipopolysaccharide injection, the survival was of 100% ($n = 12$).

4. Discussion

In this study fenspiride reduced a number of responses to lipopolysaccharide, including TNF release, alveolar neutrophil recruitment, priming of alveolar macrophages for an enhanced capacity to release arachidonic acid metabolites and the increased accumulation of extracellular type II phospholipase A_2 in serum. Fenspiride also prevented the mortality induced by a lethal dose of lipopolysaccharide.

The biological effect of lipopolysaccharide is considered to be mediated through its hydrophobic moiety, termed lipid A and covalently linked to the polysaccharide chain (Raetz et al., 1991). CD_{14}, a cell surface phosphatidylinositol-anchored glycoprotein has been recently identified as a high affinity receptor for lipopolysaccharide, following binding of the latter to lipopolysaccharide-binding protein, an acute phase protein present in serum (Tobias et al., 1992). The lipopolysaccharide activation of macrophages via the lipopolysaccharide-binding protein/CD_{14} dependent pathway leads to gene induction followed by de novo protein synthesis (Hamilton and Adams, 1987) and lipopolysaccharide-primed stimulation is dependent on transcriptional events sensitive to actinomycin D (Glaser et al., 1990).

The ability of fenspiride to inhibit zymosan-induced pleural inflammation or the tracheal smooth muscle contraction induced by several agents has been described previously (Lima et al., 1988; Advenier, 1988) and suggest that the actions of fenspiride are not the result of specific lipopolysaccharide antagonism. Therefore, inhibition by fenspiride of early steps such as the binding of lipopolysaccharide to lipopolysaccharide-binding protein and/or to CD_{14} seems unlikely and was not explored in this study. Current knowledge suggests interference with the lipopolysaccharide-induced effect at a post-receptor level and conceivably, through the inhibition of process requiring de novo protein synthesis or through the inhibition of the activity of TNFα.

TNFα plays a major role in the host response to endotoxin, including priming or activation of leukocytes (Bauldry et al., 1991), modulation of the endothelial cell function and neutrophil recruitment (Mulligan et al., 1993; Witthaut et al., 1994), increased synthesis and secretion of type II phospholipase A_2 (Pruzanski et al., 1990). TNFα is also regarded as the predominant cytokine which mediates lipopolysaccharide-induced lethality (Beutler et al., 1985). Therefore, the observed protection of fenspiride in lipopolysaccharide-treated guinea-pigs may be secondary to an inhibition of lipopolysaccharide-induced TNFα. Failure of fenspiride to inhibit in vitro serum extracellular phospholipase A_2 activity is consistent with the proposal that fenspiride interferes with type II phospholipase A_2 through reduction of its activation by TNFα. The membrane-anchored TNFα precursor is proteolytically cleaved to yield the mature cytokine by an unknown enzyme, probably a metalloproteinase (Gearing et al., 1994). Specific inhibitors of this metalloproteinase that prevent pro-TNFα cleavage also prevent the release of TNFα into the circulation of endotoxemic rats (Gearing et al., 1994). Hence, further studies are necessary to establish whether this inhibition by fenspiride occurs at the gene level or at a latter step such as cleavage or receptor blockade. Inhibition of lipopolysaccharide-induced cytokines at post transcriptional levels has been already described for anti-inflammatory drugs such as lipoxygenase/cyclooxygenase inhibitors (Hartman et al., 1993).

Another possible explanation for the anti-inflammatory effect of fenspiride, is suggested by results showing its smooth muscle relaxant effect in vitro, that might involve a theophylline-like effect, through the inhibition of phosphodiesterase activities (Advenier, 1988). Consistent with this proposed mechanism, lipopolysaccharide-induced gene expression is known to be inhibited by high intracellular cAMP concentrations (Tannenbaum and Hamilton, 1989). Furthermore, V. Gonzalves Koatz demonstrated that the phosphodiesterase IV inhibitor rolipram and dibutyryl cAMP suppressed the lipopolysaccharide-induced neutrophil recruitment to mice lungs (personal communication). In guinea-pig alveolar macrophages, we found that fenspiride did not increase cAMP concentrations in experimental conditions where the standard phosphodiesterase inhibitor IBMX, was effective. Although phosphodiesterase isozymes vary between alveolar macrophages and tracheal smooth muscle, which might mask a specific effect in phosphodiesterase activity, it seems unlikely that fenspiride operates via inhibition of phosphodiesterase or activation of adenylylcyclase activities. On the other hand, clinical trials revealed an increased therapeutic value of fenspiride after a long term administration (Fraysse et al., 1988), suggesting a target involving protein synthesis analogous to glucocorticoids action and/or accumulation during inflammation process.

We conclude that fenspiride inhibits the major effects induced in vivo by lippolysaccharide through an unknown mechanism that may be secondary to the inhibition of the synthesis or activity of TNFα.

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