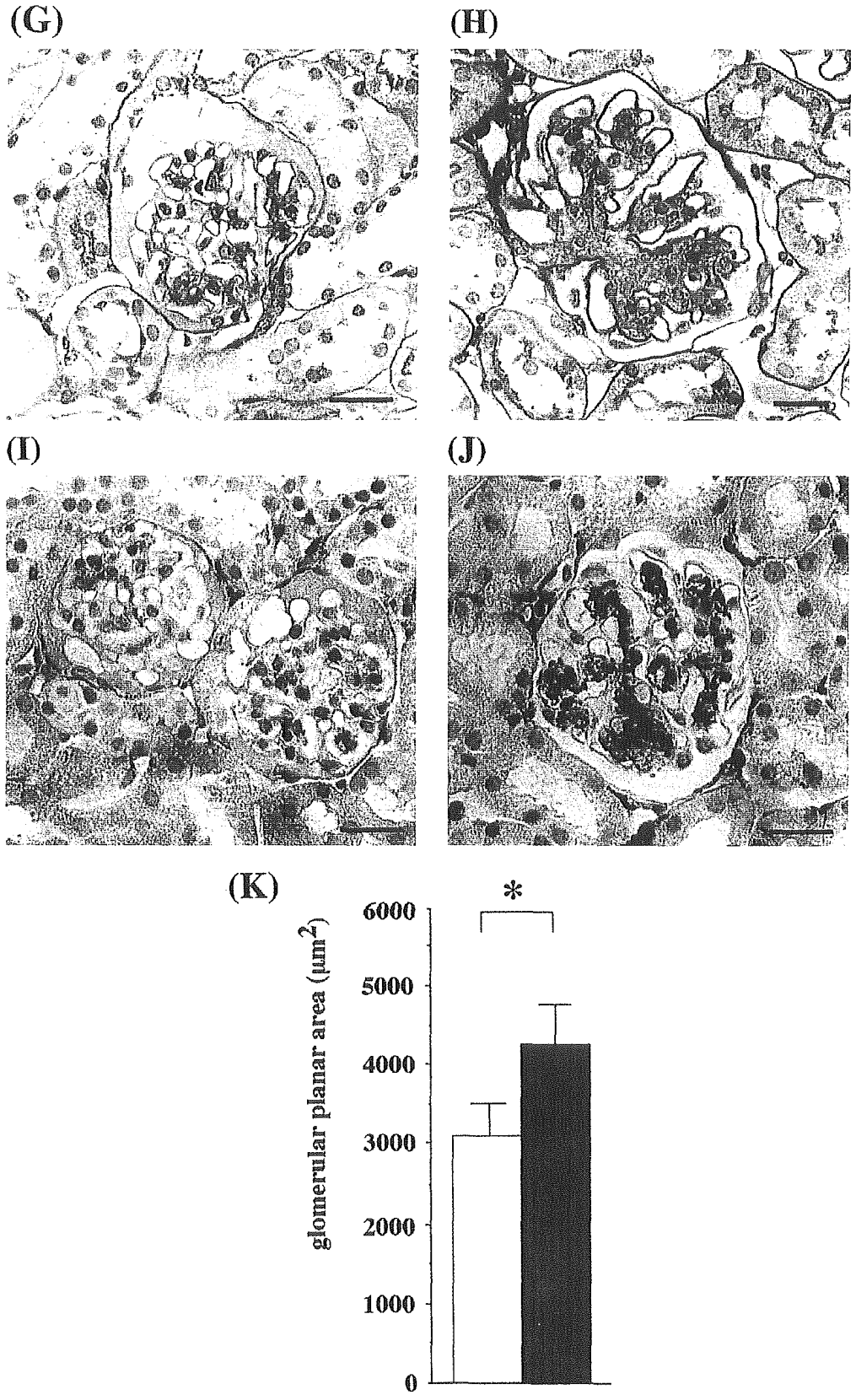
Fig. 5G–K Legend see page 111



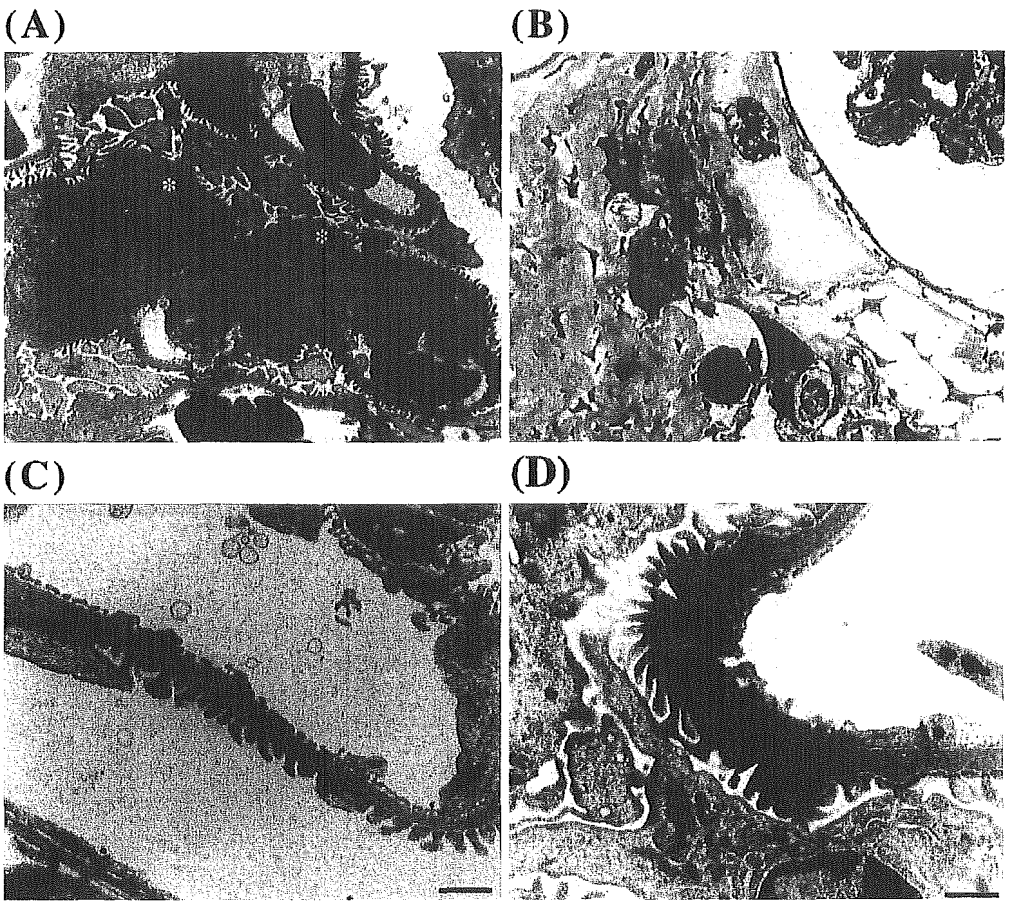


Fig. 6 Electron microscopic examination of transgenic (**A**, **B**, **D**) and wild-type (**C**) mice. *Asterisks* indicate increased mesangial matrix (**A**) or hyalinization of the interstitium (**B**). **D** *Arrowheads* indicate thickening of the basement membrane in transgenic mice. *Scale bars* in **A**, **C** and **D** 1 μm; **B** 2 μm

660±13 bpm) (Fig. 3A, B). In addition, there was no significant difference in the creatinine clearance between these mice. (wild-type: 5.1±0.4 $ml \cdot min^{-1} \cdot kg^{-1}$, transgenic: 5.0±0.3 $ml \cdot min^{-1} \cdot kg^{-1}$, n=7 in each).

Histological examination of young mice

Histological examination of the hearts (Fig. 4A, B), kidneys (Fig. 4C, D) and aortas (Fig. 4E, F) of 8- to 10-week-old ET-1 transgenic and wild-type mice revealed no apparent lesions in the transgenic mice. In particular, neither atherosclerosis of the aortic wall nor cardiac hypertrophy was noted.

Histological examination of aged mice

When aged (12 months old) transgenic mice of Tg 53 and Tg 37 were later examined, cardiac hypertrophy and atherosclerotic lesions of the aortic wall were still not detected (data not shown); however, prominent changes were found in the kidney in both lines. Numerous cystic lesions were detected in the aged transgenic mice, as were areas of focal interstitial fibrosis (Fig. 5B). The cystic lesions could be classified into three types: (1) renal cysts (planar area, 110,320±2984 μm^2) derived from the occlusion and dilatation of urinary tubules (Fig. 5C); (2) focal assemblies of small cysts (planar area, 819±104 μm^2), which were mainly observed in areas containing pronounced interstitial fibrosis, forming a wedge-shaped distribution at the renal cortex (Fig. 5D, E); and (3) glomerular cysts (planar area, 12,152±924 μm^2) (Fig. 5F). Some of the Bowman's capsules showed abnormal dilatation, and that the glomerular tufts of such glomeruli were shrunken. Comparison of the glomeruli of transgenic and wild-type mice also revealed increased numbers of mesangial cells and the accumulation of mesangial matrix in transgenic mice (Fig. 5H) and glomerular planar areas were significantly larger in transgenic mice (wild-type, 3095±450 μm^2; transgenic, 4240±486 μm^2, $P<0.01$) (Fig. 5K). In addition, PAS staining showed the basement membranes of the glomeruli from transgenic mice to be thickened and wrinkled (Fig. 5H). Immunohistochemistry of fibronectin revealed strong positive staining in the mesangial area of the glomeruli from transgenic mice (Fig. 5J), whereas no staining was detected in wild-type mice (Fig. 5I).

Electron microscopic examinations

The presence of increased mesangial matrix in the glomeruli and hyalinization of the interstitium of transgenic mice were confirmed by electron microscopy (Fig. 6A, B), as was the partial thickening of the basement membrane (Fig. 6D). In contrast, there was no apparent thick-

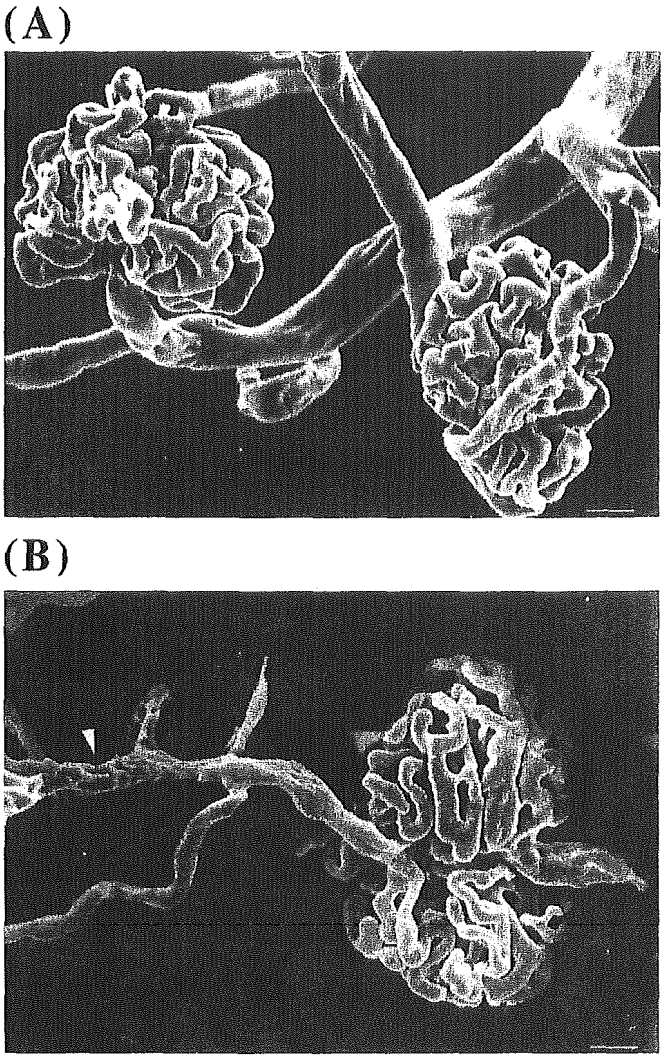


Fig. 7 Scanning electron microscopy of corrosion casts of the renal cortex of wild-type (**A**) and transgenic (**B**, **C**) mice. **B** *Arrowhead* indicates the uneven surface of the afferent arteriole in a transgenic mouse. **C** *Asterisks* indicate the cast-free space, and *arrows* indicate the club-shaped disruption of the cast in the glomerular area of a transgenic mouse. *Scale bars* 10 μm

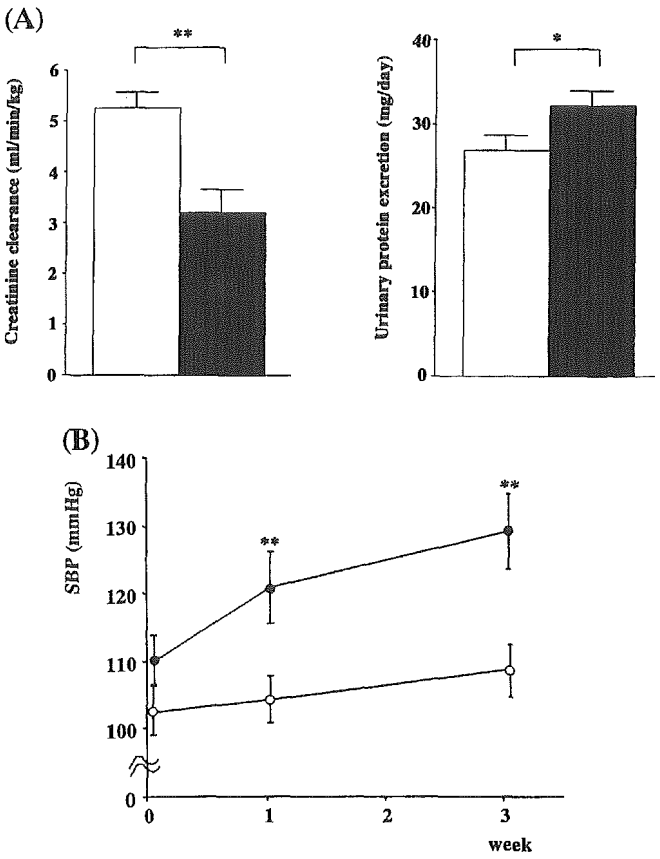


Fig. 8 **A** Creatinine clearance and urinary protein excretion of 12-month-old ET-1 transgenic (*open column*) and wild-type (*filled column*) mice (n=10 for each). **B** Time-dependent changes in BP measured in 12-month-old wild-type (*open circles*) and transgenic (*closed circles*) mice (n=10 for each) on a high-salt (8% NaCl) diet. (*SBP* Systolic blood pressure.) $^{**}P<0.01$, $^{*}P<0.05$ versus wild-type mice

ening of the basement membranes of wild-type mice (Fig. 6C).

Corrosion casts of the renal cortex

In wild-type mice, there was no narrowing or corrugation of the cast at the renal afferent and efferent arterioles; instead the surface of the cast was smooth. In areas of glomeruli, the cast was uniform in diameter and packed with complicated networks representing glomerular tufts. There were no interruptions in the cast, no clefts and no cast-free areas among the glomeruli (Fig. 7A).

In transgenic mice, by contrast, the diameter of the cast was reduced at the afferent and efferent arterioles, and its surface was uneven (Fig. 7B). Similarly, in areas of glomeruli, the cast was also irregular and reduced in diameter. Moreover, club-shaped disruptions of the cast, representing the complete occlusion of glomerular tufts, were also noted. The cast-free space was quite prominent in some glomerular areas, and might represent the hyalinization of the glomeruli (Fig. 7C).

Renal damage and salt-induced blood pressure elevation in ET-1 transgenic mice

Creatinine clearance was significantly lower in 12-month-old ET-1 transgenic mice than in wild-type mice (wild-type, 5.2±0.3 $ml \cdot min^{-1} \cdot kg^{-1}$; transgenic, 3.2±0.4 $ml \cdot min^{-1} \cdot kg^{-1}$; n=10 in each; P<0.01), and total urinary protein excretion was significantly higher in the aged transgenic mice (wild-type, 26.9±1.7 mg/day; transgenic, 32.9±1.8 mg/day; n=10 in each; P<0.05) (Fig. 8A).

When a high-salt (8% NaCl) diet was provided to 12-month-old transgenic and wild-type mice for 3 weeks, the wild-type mice showed only a slight tendency toward elevation of systolic BP. On the other hand, the systolic BP of the 12-month-old transgenic mice was significantly increased at 1 week of initiating the high-salt diet (Fig. 8B).

Discussion

By the time they were about 12 months old, ET-1 transgenic mice exhibited pronounced interstitial fibrosis, renal cysts and glomerulosclerosis, changes similar to those seen by Hocher et al. in their model [16]. Renal function, as estimated from the glomerular filtration rate, was also diminished in transgenic mice, and the increased urinary protein excretion was indicative of glomerular dysfunction. It is unlikely that the renal damage was a consequence of systemic hypertension, because the elevation of BP was significant only after high-salt loading. Electron microscopy of corrosion casts revealed the lumens of the renal afferent and efferent arterioles and the glomerular tufts to be narrowed and uneven in transgenic mice, and some of the glomeruli to be completely occluded. Vascular casting revealed similar changes in spontaneously hypertensive rats infused with ET-1 [17], making it conceivable that the renal damage was caused by local hemodynamic and arteriosclerotic changes induced by ET-1 overexpression that did not primarily affect systemic BP. With respect to glomerular filtration, ET-1 contracts afferent and efferent arterioles equally, but some evidence suggests that endogenous ET-1 predominantly affects efferent arterioles [18], which would be expected to induce intraglomerular hypertension, resulting in glomerular damage.

Nonvascular effects of ET-1 may also contribute to both the glomerulosclerosis and the interstitial fibrosis. For example, ET-1 has a potent proliferative effect on a variety of cell types, including glomerular mesangial cells [19, 20], and it promotes the synthesis of extracellular matrix [21, 22]. As mesangial and epithelial cells respectively produce mesangial and basement membrane matrices, ET-1 overproduction may directly act on these cells and cause glomerulosclerosis. Indeed, proliferation of mesangium and thickening of the basement membrane were detected by electron microscopy in the glomeruli of transgenic mice.

Aged transgenic mice typically developed three types of cystic lesions in the kidney: renal cysts, focal assemblies of small cysts and glomerular cysts. Interestingly, it was recently reported that the kidneys of Han:SPRD rats, a well-known model of human polycystic kidney disease, exhibited significantly higher levels of ET-1 than age-matched controls [23]. Thus, elevated renal ET-1 may itself contribute to cyst formation in the kidney. The renal cysts were probably a consequence of the occlusion and dilatation of the urinary tubules, but it remains unclear why the occlusion of urinary tubules was so frequent. One possibility is that the pronounced interstitial fibrosis disturbed urine passage through the urinary tubules.

On the other hand, focal assemblies of small cysts in the kidney were unique to our model. These lesions were clearly different from what are more commonly described as renal cysts, since they did not exhibit dilatation, and they were not lined with cells. Nonetheless, because degenerated cuboid cells, resembling urinary tubular cells, were scattered around them, we believe these cysts to be derived from the urinary tubules. Moreover, as the distribution of these cysts was wedge-shaped, and mainly located in regions of pronounced interstitial fibrosis, we suggest that the degeneration of the urinary tubules may have been caused by intrarenal ischemia.

Dilatation of Bowman's capsule and glomerular cysts were also detected in transgenic mice. Similar cystic lesions were observed in a chronic glomerulonephritis model induced in rat by administration of nephritogenic glycoprotein [24]. Hence, when chronically present at high levels, ET-1 may act as a nephritogenic factor.

By 12 months of age, ET-1 transgenic mice had developed salt-dependent hypertension. The observed salt-dependent hypertension would be secondary to a loss of functional units for Na^+ excretion by the kidney. It has been postulated that the progression of glomerulosclerosis exacerbates hypertension by reducing renal mass, thereby reducing the kidney's capacity to excrete Na^+.

Research into the function of ET-1 entered a new era with the clinical application of ET-1 antagonists. However, accurate estimation of the efficacy of anti-ET-1 therapy in treating human disease will require continued accumulation of both clinical and preclinical data. The present study clearly showed that mild, chronic overproduction of ET-1 is detrimental to the kidney. In the context of the pathophysiology of hypertension, ET-1 may work as a mediator, facilitating the development of renal damage, which would then exacerbate elevations in BP. If so, ET-1 antagonists may serve to attenuate hypertension-induced organ damage.

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Role of the platelet-activating factor (PAF) receptor during pulmonary infection with gram negative bacteria

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1 The lipid mediator PAF plays an important role in the phagocytosis of particles, including bacteria, and consequent production of pro-inflammatory cytokines, such as TNF-α and IL-8.

2 Using a PAF receptor antagonist (UK-74,505) and PAF receptor knock-out mice, we have investigated the relevance of PAF for the inflammatory changes and lethality after pulmonary infection with the gram-negative bacteria *Klebsiella pneumoniae* in mice.

3 At an inoculum of 3×10^6 bacteria, there was marked pulmonary (bronchoalveolar lavage and lung) neutrophilia that started early (2.5 h after infection) and peaked at 48 h. All animals were dead by day 4 of infection. The chemokine KC and the pro-inflammatory cytokine TNF-α increased rapidly and persisted for 48 h in the lungs.

4 Pretreatment with UK-74,505 (30 mg kg^{-1} per day, p.o.) had no significant effects on the number of infiltrating neutrophils in BAL fluid or lung tissue, as assessed by histology and measuring myeloperoxidase, or on the concentrations of KC. In contrast, concentrations of TNF-α and the number of bacteria inside neutrophils were significantly diminished.

5 In order to support a role for the PAF during *K. pneumoniae* infection, experiments were also carried out in PAFR-deficient mice. In the latter animals, lethality occurred earlier than in wild-type controls. This was associated with greater number of bacteria in lung tissue and diminished percentage of neutrophils containing bacteria in their cytoplasm.

6 Our results suggest that PAF, acting on its receptor, plays a protective role during infection with *K. pneumoniae* in mice.

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Keywords: Chemokines; lung inflammation; neutrophils; TNF-α; recruitment

Abbreviations: BAL, bronchoalveolar lavage; CFU, colony forming units; KC, keratinocyte-derived chemokine; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MPO, myeloperoxidase; PAF, platelet activating factor; PAFR, PAF receptor; $PAFR^{-/-}$, PAF receptor-deficient mice; PBS, phosphate buffered saline; PMN, polymorphonuclear leukocyte; TNF-α, tumour necrosis factor-α

Introduction

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a potent autacoid lipid mediator with various biological activities, including platelet and leukocyte activation. PAF acts by binding to a G protein-coupled seven transmembrane receptor, the PAF receptor (PAFR), and appears to regulate constitutively various physiological processes (Ishii & Shimizu, 2000). In addition to its role as a physiological mediator, PAF has been shown to play an important role in the pathophysiology of various inflammatory conditions (Ishii & Shimizu, 2000). Studies with PAFR antagonists or PAFR-deficient animals have shown an essential role of PAFR during systemic allergic anaphylaxis-associated shock in mice (Ishii & Shimizu, 2000; Montrucchio *et al.*, 2000). Studies with PAFR antagonists or strategies that decrease PAF activity have also demonstrated an important role of PAF for lipopolysaccharide (LPS)-induced shock and lethality (e.g. Fukuda *et al.*, 2000) and for the lung injury which follows a range of inflammatory stimuli (Miotla *et al.*, 1998; Tavares-de-Lima *et al.*, 1998; De Matos *et al.*, 1999).

More recently, we have suggested an important role of PAFR for the protective immune response of the murine host against infection with an intracellular protozoan parasite, *T. cruzi* (Aliberti *et al.*, 1999). In the latter system, PAF induced NO release by *T. cruzi*-infected macrophages *in vitro* and pretreatment of mice with PAFR antagonists increased blood parasitaemia and enhanced infection-associated lethality (Aliberti *et al.*, 1999). These results are in line with the ability of leukocytes to produce PAF upon encounter with microorganisms or soluble particles and to engulf them in a PAF-dependent manner (Makristathis *et al.*, 1993; Au *et al.*, 2001). Moreover, exposure of leukocytes to endotoxin or bacteria may trigger PAF release (reviewed by Montrucchio *et al.*, 2000; Makristathis *et al.*, 1993). Thus, it is clear that PAF may have a dual role during bacterial

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infections. On one hand, PAF appears to play an important role in the ability of a host to deal with infections by facilitating phagocytosis and killing of engulfed microorganisms. On the other hand, PAFR activation may underlie the tissue injury and shock associated with the infection and endotoxin released.

In this study, we have investigated the relevance of PAF receptors in a model of pulmonary infection in mice caused by gram-negative bacteria. Thus, we have assessed the effects of the treatment with a PAFR antagonist, UK-74,505, on the lethality bacterial counts and inflammatory indices following pulmonary infection of mice with *Klebsiella pneumoniae*. UK-74,505 is a potent, specific, orally available and long-acting PAFR antagonist (Alabaster *et al.*, 1991; Parry *et al.*, 1994; Jezequel *et al.*, 1996). For comparison, we also assessed the lethality and infection indices of PAFR$^{-/-}$ mice after infection with *K. pneumoniae*.

Methods

Animals

Balb/C (8 to 12 week-old) female mice obtained from the Bioscience unit of our Institution were housed in standard conditions and had free access to commercial chow and water. PAF receptor-deficient (PAFR$^{-/-}$) mice were generated as previously described and backcrossed or at least 10 generations into a Balb/C background (Ishii *et al.*, 1998). All procedures described here had prior approval from the animal ethics committee of Instituto de Ciências Biológicas (Belo Horizonte, Brazil).

Bacteria

The bacterium used was *Klebsiella pneumoniae* – ATCC 27 736 that has been kept in the Department of Microbiology, Universidade Federal de Minas Gerais. Before the experiments described herein, bacteria were made pathogenic by 10 passages in Balb/C mice (i.p. injection and collection in the spleen 24 h later) and kept frozen in a −70°C freezer at a concentration of 1×10^9 CFU ml^{-1} in tryptic soy broth containing 10% glycerol (v v^{-1}) until use. Bacteria were frozen when in the log phase of growth.

Treatment with UK-74,505

The PAF receptor antagonist UK-74,505 (modipafant, a gift of Dr J. Parry, Pfizer, Sandwich, U.K.) was dissolved initially in 0.1 M HCl and further diluted 10 fold in saline. Control animals received an oral administration of vehicle (0.01 M HCl), whereas the test group received an oral administration of UK-74,505 at dose of 30 mg kg^{-1}. The oral dose chosen was recommended by the supplier and has been previously shown to give good bioavailability for 24 h (Alabaster *et al.*, 1991; Parry *et al.*, 1994; Jezequel *et al.*, 1996). For lethality experiments, the drug was administered 24 and 2 h prior to inoculation of bacteria and daily thereafter. For the experiments measuring infection and inflammatory indices, the drug was administered 24 and 2 h prior to inoculation of bacteria and animals sacrificed 24 h after inoculation.

K. pneumoniae *inoculation*

K. pneumoniae was grown in tryptic soy broth (Difco, Detroit, MI, U.S.A.) for 18 h at 37°C prior to inoculation. The concentration of bacteria in broth was routinely determined by serial 1 : 10 dilutions. One hundred microlitres of each dilution were plated on McConkey agar plates and incubated for 24 h at 37°C and then colonies were counted. Each animal was anaesthetized i.p. with 0.2 ml of a solution containing xylazin (0.002 mg ml^{-1}), ketamin (50 mg ml^{-1}) and saline in a proportion of 1 : 0.5 : 3, respectively. The trachea was exposed and 30 μl of a suspension containing 3×10^6 *K. pneumoniae* or saline was administered with a sterile 26-gauge needle. The skin incision was closed with surgical staples.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed to obtain leukocytes in the alveolar spaces. The trachea was exposed and a 1.7-mm-outside-diameter polyethylene catheter inserted. BAL was performed by instilling three 1-ml aliquots of PBS and approximately 2 ml of fluid was retrieved per mouse. The number of total leukocytes was determined by counting leukocytes in a modified Neubauer chamber after staining with Turk's solution. Differential counts were obtained from cytospin preparations by evaluating the percentage of each leukocyte on a slide stained with May-Grunwald-Giemsa. In some experiments, the percentage of BAL neutrophils that had phagocytosed at least one bacterium was evaluated in at least 200 cells.

Determination of myeloperoxidase activity

The extent of neutrophil accumulation in the lung tissue was measured by assaying myeloperoxidase activity as previously described (De Matos *et al.*, 1999). Using the conditions described below, this methodology is very selective for the determination of neutrophils over macrophages (data not shown). Briefly, a portion of left lungs of animals was removed and snap frozen in liquid nitrogen. Upon thawing, the tissue (0.1 g of tissue per 1.9 ml of buffer) was homogenized in pH 4.7 buffer (0.1 M NaCl, 0.02 M $NaPO_4$, 0.015 M NaEDTA), centrifuged at $3000 \times g$ for 10 min and the pellet subjected to hypotonic lyses (1.5 ml of 0.2% NaCl solution followed 30 s later by addition of an equal volume of a solution containing NaCl 1.6% and glucose 5%). After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and re-homogenized. One millilitre aliquots of the suspension were transferred into 1.5 ml-Eppendorf tubes followed by three freeze-thaw cycles using liquid nitrogen. The aliquots were then centrifuged for 15 min at $3000 \times g$, the pellet was resuspended to 1 ml and samples of lung were diluted (1 : 20) prior to assay. Myeloperoxidase (MPO) activity in the resuspended pellet was assayed by measuring the change in optical density (O.D.) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.5 mM). Results were expressed as 'myeloperoxidase index' and were calculated by comparing the O.D. of tissue supernatant with the O.D. of mouse peritoneal neutrophils processed in the same way. To this

end, neutrophils were induced in the peritoneum of mouse by injecting 3 ml of casein 5%. A standard curve of neutrophil (>95% purity) numbers versus O.D. was obtained by processing purified neutrophils as above and assaying for MPO activity.

Determination of plasma and lung K. pneumoniae *colony forming units*

At time of sacrifice, plasma was collected from the branchial plexus, the right ventricle was perfused with 3 ml of sterile saline and lungs were harvested. Tissues were then homogenized with a homogenizer in a vented hood. The homogenates and plasma were placed on ice, and serial 1:10 dilutions were made. One hundred microlitres of each dilution were plated on McConkey agar plates (Difco) and incubated for 24 h at 37°C and then the number of colony forming units (CFU) was counted. The detection limit of the assay was 100 bacteria ml^{-1} or 100 bacteria per 100 mg of tissue.

Harvesting of lungs and blood for cytokine analysis

At the designated time point, mice were anaesthetized with xylazin/ketamin/saline as above, blood collected from the brachial plexus and the animals sacrificed. Prior to lung removal, the pulmonary vasculature was perfused with 3 ml of PBS *via* the right ventricle. The right lung was then harvested for assessment of the various cytokine protein levels.

Measurement of cytokine concentrations in serum, BAL and lungs

The cytokine concentrations (TNF-α, KC, MCP-1/JE) were measured in serum, BAL and lung of animals using ELISA techniques with commercially available antibodies and according to the instructions supplied by the manufacturer (R&D Systems). Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at −20°C until further analysis. Serum and BAL samples were analysed at a 1:3 and 1:5 dilution in assay dilution buffer, respectively. One hundred milligrams of lung of controls and treated animals were homogenized in 1 ml of PBS (0.4 m NaCl and 10 mM $NaPO_4$) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at $3000 \times g$ and the supernatant immediately used for ELISA assays at a 1:5 dilution in assay dilution buffer. The detection limit of the ELISA assays was 16 pg ml^{-1}.

Determination of the levels of circulating leukocytes

The total number of circulating leukocytes and neutrophils were evaluated in blood samples obtained at the end of the experiments described in Figure 1. The number of total circulating leukocytes was determined by counting leukocytes in a modified Neubauer chamber after staining with Turk's solution and differential counts by evaluating the percentage of each leukocyte on blood films stained with May-Grunwald-Giemsa.

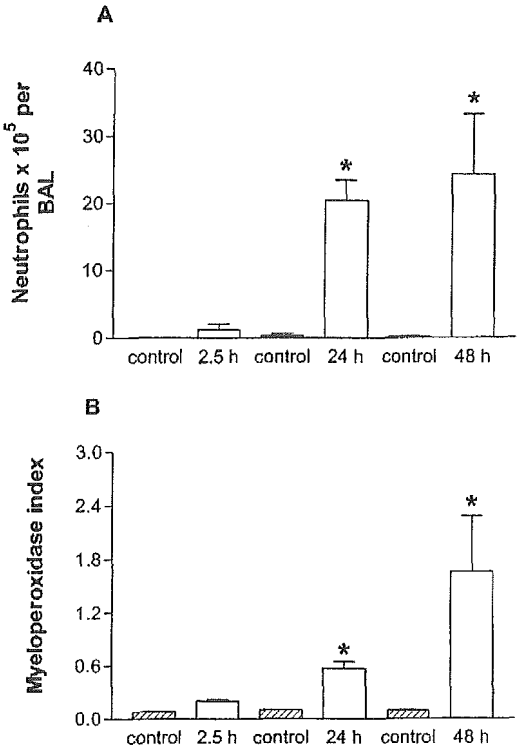

Figure 1 Kinetics of the influx of neutrophils in the lungs of mice infected with *K. pneumoniae*. Animals were inoculated with 3×10^6 bacteria or vehicle (30 μl) and neutrophil influx in (A) in the bronchoalveolar lavage (BAL) fluid or (B) lungs assessed after 2.5, 24 and 48 h. Myeloperoxidase (MPO) activity in lungs was used as an index of neutrophil influx in that tissue. Results are shown as the number of neutrophils or leukocyte index and represent the mean±s.e.mean of six animals in each group. $^*P<0.01$ when compared with uninfected animals.

Histology

Lungs were inflated with 2 ml phosphate-buffered 10% formalin, embedded in paraffin and 4 μm-thick sections obtained. The sections were then stained with haematoxylin and eosin and examined under a light microscope.

Statistical analysis

Results are shown as means±s.e.mean. Data sets were compared by using analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* analysis. Results were considered significant when $P<0.05$.

Results

Kinetics of the pulmonary inflammation and infection after intratracheal (i.t.) inoculation of K. pneumoniae

Initial experiments were carried out to characterize the kinetics of the pulmonary response after instillation of *K. pneumoniae*. Mice were injected with 30 μl of a saline suspension containing 3×10^6 bacteria, an inoculum shown to be optimal for inducing lung inflammation and survival of animals for at least 48 h (data not shown). Twenty-four

hours after inoculation of the bacteria, animals developed signs of infection – lethargy, decreased food intake, piloerection and ruffled fur. Histological analysis showed a marked diffuse infiltration of neutrophils in the lungs of infected animals. Many neutrophils were found in the alveolar spaces and occasional neutrophil aggregates were observed. In addition, there was diffuse hyperaemia and scattered areas of haemorrhage (data not shown). Within the time frame of the experiment (48 h) and when compared to uninfected mice, there did not seem to be an increase in the number of mononuclear cells in lung tissues (data not shown). In order to quantify the influx of neutrophils into the lungs of infected animals, we assessed the number of neutrophils (PMN) in BAL fluid and tissue MPO activity. In BAL fluid, there was an accumulation of PMN that was substantial at 24 h and was sustained until 48 h after infection (Figure 1A). Similarly, there was a marked infiltration of neutrophils in the lung parenchyma, as assessed by tissue MPO activity (Figure 1B). Neutrophils were first observed at 24 h after infection and greater numbers were observed after 48 h (Figure 1B). With an exception of a neutrophilia observed at 2.5 h, there were few modifications in total or differential leukocyte counts in blood (data not shown).

The concentration of bacteria in the lungs of infected mice rapidly increased after the inoculation of *K. pneumoniae* (Figure 2). The total number of bacteria was already greater than 1×10^8 CFU per lung 2.5 h after infection. These concentrations increased rapidly and were not countable at 48 h at the dilutions used (Figure 2). In contrast, we failed to observe any dissemination of the infection, as no *K. pneumoniae* colony could be determined in plasma in any of the time points until 48 h after infection (data not shown).

KC and TNF-α are known to play a role during gram negative bacterial infection in mice (Tsai *et al.*, 1998; Greenberger *et al.*, 1995; Laichalk *et al.*, 1996). Experiments evaluating the kinetics of expression of KC and TNF-α in lung homogenates after *K. pneumoniae* are shown in Figure 3. Markedly elevated concentrations of both cytokines in lungs were already detectable at 2.5 h after infection. The concentrations were still elevated at 24 h and levels started to fall at 48 h (Figure 3). No TNF-α could be detected in plasma samples in any of the time points measured and KC

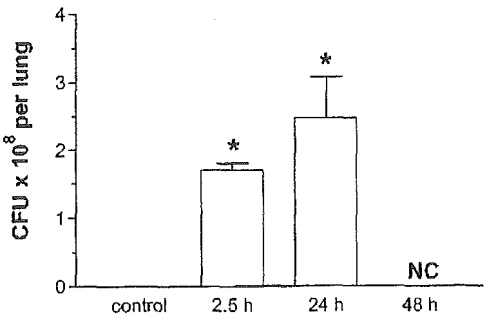

Figure 2 Kinetics of the number of colony-forming units (CFU) in the lungs of mice infected with *K. pneumoniae*. Animals were inoculated with 3×10^6 bacteria or vehicle (30 μl and the number of CFU in lung tissue evaluated after 2.5, 24 and 48 h. At 48 h, the number of CFU was not countable (NC). Results are shown as the mean±s.e.mean of five animals in each group. $^*P<0.01$ when compared with uninfected animals.

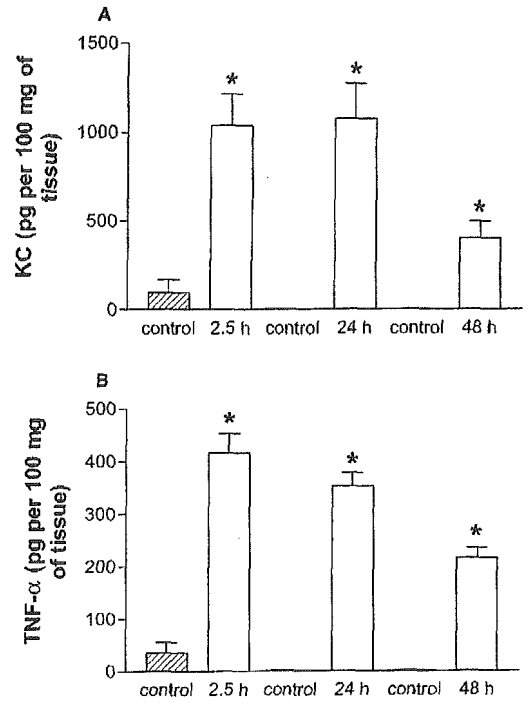

Figure 3 Kinetics of TNF-α and KC protein production in lung homogenates after infection with *K. pneumoniae*. Animals were inoculated with 3×10^6 bacteria or vehicle (30 μl) and the concentration of KC (A) and TNF-α (B) in lung tissue evaluated by ELISA after 2.5, 24 and 48 h. Results are shown as the mean±s.e.mean of six animals in each group. $^*P<0.01$ when compared with uninfected animals.

was detectable in plasma at 2.5 h and 24 h after infection but not at 48 h (control, below detection limit; 2.5 h, 759.35 ± 209.95 pg ml^{-1}; 24 h, 121.4 ± 92.0 pg ml^{-1}; 48 h, below detection limit; $n=6$). In all further experiments, samples were harvested at 24 h after infection, as tissue inflammation was marked at this time point, bacteria levels in tissues were elevated and all animals were alive but with signs of infection.

Effects of the treatment with the PAF receptor antagonist UK-74,505 on the course of K. pneumoniae *infection*

Treatment with UK-74,505 had no significant effect on the number of neutrophils which were recruited into the airspaces of infected animals (infected animals, $34.0 \pm 17.7 \times 10^5$ neutrophils; infected and UK-74,505-treated, $42.8 \pm 13.7 \times 10^5$, $n=6$). Similarly, the recruitment of neutrophils in the lung of UK-74,505-treated mice, as assessed by MPO assay (Infected, 1.2 ± 0.2; Infected + UK-74,505, $0.9 \pm 0.2 \times 10^6$ neutrophils 100 mg^{-1} of tissue, $n=6$), was not significantly different from vehicle-treated *K. pneumoniae*-infected animals. Histological analysis of lungs of infected animals treated with vehicle or UK-74,505 showed a marked diffuse infiltration of neutrophils, hyperaemia and areas of haemorrhage. There did not appear to be any qualitative differences between the two groups (data not shown).

In agreement with the lack of effect of the PAFR antagonist on neutrophil recruitment into the lung tissue, the tissue and BAL fluid concentrations of the neutrophil-

active chemokine KC was not different in UK-74,505-treated and untreated mice (Figure 4A,B). Similarly, pulmonary tissue concentrations of MCP-1 were not significantly different in both groups of animals (Infected, 1516.0±194.0 pg per 100 mg of tissue; infected and UK-74,505-treated, 1129.4±103.4 pg per 100 mg of tissue; $n=6$). In contrast, treatment with UK-74,505 significantly decreased the concentrations of TNF-α detected in the lung and BAL fluid of *K. pneumoniae*-infected mice (Figure 4C,D).

The effects of daily treatment of *K. pneumoniae*-infected mice with UK-74,505 on bacterial counts is shown in Figure 5. Treatment with UK-74,505 resulted in a significant increase in the number of CFU in the lungs of *K. pneumoniae*-infected mice (Figure 5A). Similarly, infection of PAFR$^{-/-}$ mice with *K. pneumoniae* resulted in a larger number of CFU in the lungs when compared to wild-type controls (Figure 5B).

The percentage of BAL neutrophils that had ingested at least one bacterium was evaluated 24 h after infection. As seen in Table 1, pre-treatment with UK-74,505 was accompanied by a 50% inhibition of the ability of BAL neutrophils to phagocytose *K. pneumoniae*. Similarly, there was a marked suppression of *K. pneumoniae* uptake by BAL neutrophils from PAFR$^{-/-}$ mice when compared to their wild type controls (Table 1).

Survival of mice infected with K. pneumoniae

Subsequent experiments were performed to examine the contribution of PAF to the survival of mice infected with *K. pneumoniae*. As shown in Figure 6, all untreated *K. pneumoniae*-infected animals were alive by 72 h of infection after which time mortality increased substantially, with 100% lethality noted by day 5 after inoculation. The treatment with UK-74,505 resulted in earlier lethality with 30 and 85% of animals dead at 72 and 96 h, respectively (Figure 6). Similarly, infection of PAFR$^{-/-}$ mice with *K. pneumoniae* resulted in significantly earlier lethality when compared to wild-type animals (Figure 6). Of note, 40% of animals were dead at 48 h after infection.

Discussion

The host defence against acute bacterial infection requires the generation of a vigorous inflammatory response that predominantly involves recruitment and activation of neutrophils. Although in the majority of cases this response is sufficient to control infection, an overt inflammatory response may cause marked tissue injury, haemodynamic shock and death (Teixeira *et al.*, 2001). PAF is a biologically active phospholipid mediator known to be important for the ability of leucocytes to phagocytose foreign particles and kill microorganisms (Ishii & Shimizu, 2000; Au *et al.*, 2001). On the other hand, several studies have demonstrated the role of PAF and its receptor in mediating tissue injury, shock and lethality following endotoxin challenge and other acute inflammatory stimuli (Miotla *et al.*, 1998; Fukuda *et al.*, 2000; Ishii and Shimizu, 2000; Montrucchio *et al.*, 2000; Souza *et al.*, 2000). Thus, during acute bacterial infection,

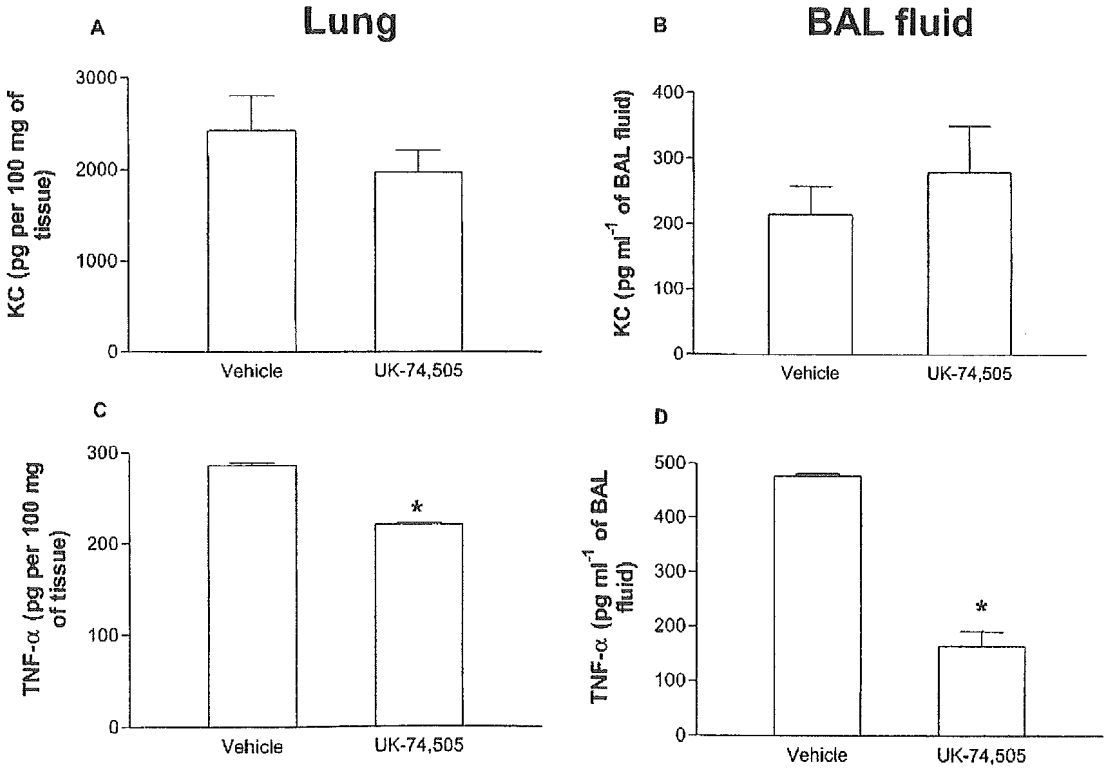

Figure 4 Effect of pre-treatment with the PAF receptor antagonist UK 74,505 on the production of KC and TNF-α after infection with *K. pneumoniae*. Animals were inoculated with 3×10^6 bacteria or vehicle (30 μl) and the concentration of KC (A,B) and TNF-α (C,D) in lung tissue (A,C) or BAL fluid (B,D) evaluated by ELISA after 24 h. Results are shown as the mean±s.e.mean of six animals in each group. *$P<0.01$ when compared with animals treated with vehicle.

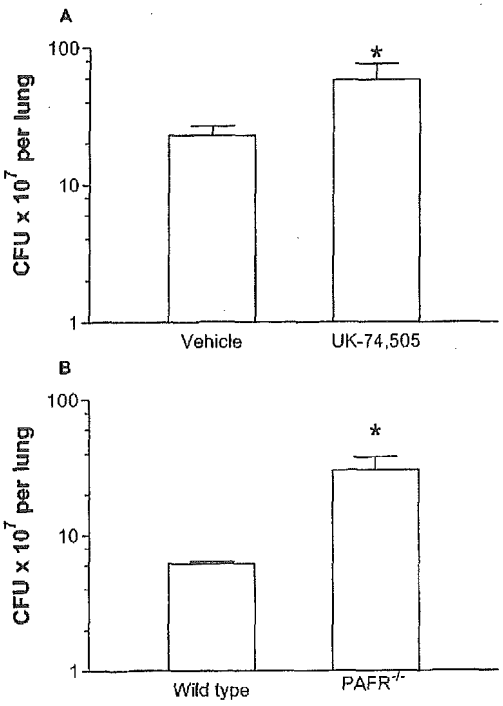

Figure 5 Number of colony-forming units (CFU) after infection with *K. pneumoniae* (A) after the pre-treatment with the PAF receptor antagonist UK 74,505 or in (B) $PAFR^{-/-}$ mice. Animals were inoculated with 3×10^6 bacteria or vehicle (30 μl) and the number of CFU in lung homogenates evaluated after 24 h. $PAFR^{-/-}$ mice were compared to their respective wild-type controls. Results are shown as the mean ± s.e.mean of five animals in each group. $^*P < 0.01$ when compared with animals treated with vehicle-treated wild-type controls.

Table 1 Percentage of neutrophils from bronchoalveolar lavage of *K. pneumoniae* infected mice containing at least one bacterium in the cytoplasm

Groups	*% neutrophils containing bacteria*
Wild type or vehicle treated	62.2 ± 3.3
UK-74,505 treated	31.1 ± 6.3*
$PAFR^{-/-}$	10.1 ± 5.1*

Animals were inoculated with 3×10^6 bacteria or vehicle (30 μl) and the percentage of neutrophils containing at least one bacterium in the cytoplasm was assessed. Results are shown as the mean ± s.e.mean of 3–6 animals in each group. $^*P < 0.01$ when compared with animals treated with vehicle-treated wild-type controls.

activation of PAFR may be important for the ability of the host to deal with infection but could underlie or contribute to the systemic inflammatory response observed in the most severe cases. These possible dual effects of PAFR activation led us to investigate the functional role of the receptor in a model of lung infection with *K. pneumoniae*.

In our model of lung infection, we chose to use *K. pneumoniae* for several reasons. This gram-negative aerobic organism is an important cause of community-acquired pneumonia in individuals with impaired pulmonary defences and is a major pathogen for nosocomial pneumonia (Granton & Grossman, 1993; Maloney & Jarvi, 1995). Importantly, preliminary studies showed that after intratracheal (i.t.)

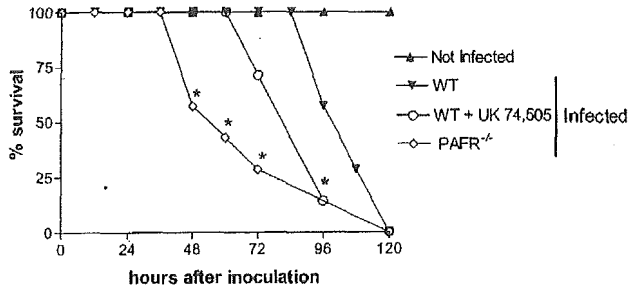

Figure 6 Role of the PAF receptor for the survival of animals after infection with *K. pneumoniae*. Four groups of animals were evaluated (eight animals in each group): infected animals (inoculated with 3×10^6 bacteria) which were PAF receptor-deficient ($PAFR^{-/-}$), wild-type (Infected WT) or wild-type treated with the PAF receptor antagonist UK 74,505 (WT + UK 74,505) and animals which received only vehicle (30 μl, not infected). The number of dead animals was evaluated every 12 h and results are shown as per cent survival. Survival of $PAFR^{-/-}$ and UK 74,505-treated animals was significantly different from that of infected WT animals ($P < 0.05$).

inoculation with *K. pneumoniae* mice developed pneumonia with features resembling human disease. Moreover, there is a reproducible relationship between the size of the inoculum and lethality of infection (Toews *et al.*, 1979). A dose of 3×10^6 bacteria was chosen for all experiments as this dose allowed for the development of substantial inflammation within 24 to 48 h without excessive mortality. Untreated animals receiving this dose of *K. pneumoniae* were unable to clear the bacteria and all animals ultimately died. Therefore, this dose allowed for assessment of the inflammatory mediators and bacterial growth, as well as the effects on survival.

In our experiments, the inoculation of *K. pneumoniae* induced a time-dependent increase of infiltration of neutrophils and pro-inflammatory cytokines in the lungs of infected mice. The number of neutrophils increased rapidly and was markedly elevated 24 h after the infection. The increase in neutrophil numbers in BAL fluid and lungs was preceded by an increase in the concentrations of TNF-α and KC. Of note, these pro-inflammatory mediators have been shown to play important roles in the control of bacterial infection and lung inflammation following pulmonary infection with *K. pneumoniae* (Laichalk *et al.*, 1996; Tsai *et al.*, 1998; 2000). Interestingly, at the inoculum used and at time points observed, the inflammatory response seemed to be mostly compartmentalized in the lungs, as the number of leukocytes, and the concentration of cytokines (with the exception of KC) and bacteria were not significantly elevated in the plasma of infected animals. Only later was the control of the infection lost and lethality occurred. Overall, our experiments are in good agreement with observations of other studies examining pulmonary inflammation after *K. pneumoniae* infection (e.g.: Laichalk *et al.*, 1996; Tsai *et al.*, 1998; 2000).

Daily treatment with the PAFR antagonist UK-74,505 had no relevant effect on the influx of neutrophils, as assessed by the number of these cells in BAL fluid, MPO activity and histological analysis of lung tissue. Previous studies have suggested a role for neutrophil-active (CXC) chemokines and chemokine receptors for the migration of neutrophils into the lungs of mice infected with bacteria (Greenberger *et al.*, 1996; Moore *et al.*, 2000; Tsai *et al.*, 2000; Fillion *et al.*, 2001). In addition to the lack of effect of PAFR blockade on neutrophil influx, UK-74,505 had little effect on the tissue

concentrations of the CXC chemokine KC. These results suggest that the early generation of PAF does not play a role in the generation of the neutrophil-active chemokine KC and subsequent sequestration of neutrophils into the lungs of infected mice. Similarly, PAF failed to affect the recruitment of neutrophils to the lungs after i.v. challenge with endotoxin (Miotla *et al.*, 1998) or after acid-induced lung injury (Nagase *et al.*, 1999) in mice. Thus, whereas PAFR activation may play a role in phagocytosis-dependent production of the CXC chemokine IL-8 after stimulation with a particulate stimulus (zymosan) (Au *et al.*, 2001), production of KC after bacterial infection *in vivo* seemed to be independent of PAFR activation.

Previous studies (Laichalk *et al.*, 1996) have shown a critical role of TNF-α as part of the pulmonary host defense in a murine model of infection with *K. pneumoniae*. In our model, there was a marked pulmonary production of TNF-α that peaked early after infection and was maximal around 24 h. Pretreatment with UK-74,505 significantly reduced the concentrations of TNF-α both in BAL fluid and pulmonary tissue extracts after *K. pneumoniae* infection. These effects of PAF on TNF-α production seemed to be of physiopathological relevance, as demonstrated by the increase in the number of CFU in the lungs of *K. pneumoniae*-infected mice. The increase in bacterial counts in the lungs were reflected in the severity of the disease, as UK-74,505-treated animals died significantly faster than vehicle-treated controls. Our lethality results are in contrast with a previously published study evaluating the effect of a distinct, shorter-acting PAFR antagonist (WEB2170) in a model of *K. pneumoniae* infection in NMRI mice (Makristathis *et al.*, 1993). In this latter study, WEB2170 treatment was accompanied by a small, dose-independent increase in survival and a marginal, dose-independent decrease in bacterial counts. Moreover, statistical analysis was not provided in that study (Makristathis *et al.*, 1993).

The role of PAFR for the ability of the host to mount an effective immune response was even more markedly appreciated when PAFR-deficient animals were used. In these animals, significant lethality was already noticeable 48 h after infection. Thus, our results clearly suggest that the ability of PAFR to modulate TNF-α production in the lungs of mice may be relevant for an effective innate immune response against *K. pneumoniae* pulmonary infection.

An important role for PAFR in the phagocytosis of particulate stimuli and ensuing pro-inflammatory cytokine production has been demonstrated in several studies (Au *et al.*, 1994, 2001; Aliberti *et al.*, 1999; Owaki *et al.*, 2000). Interestingly, the phagocytosis of *K. pneumoniae* by neutrophils is accompanied by the release of PAF from neutrophils (Makristathis *et al.*, 1993). One possibility that stems from the latter observations is that, in our model, the blockade of PAFR may have prevented the ability of phagocytes to engulf bacteria and produce pro-inflammatory cytokines, such as TNF-α, in response to the phagocytic stimulus. In this regard, results of the pre-treatment with UK-74,505 or experiments with $PAFR^{-/-}$ mice showed that fewer neutrophils that migrated to the lungs after *K. pneumoniae* infection ingested bacteria. Moreover, a role of intracellular PAF for the production of TNF-α by macrophages has been previously demonstrated *in vitro* (Yamada *et al.*, 1999; Tsuyuki *et al.*, 2002). Overall, the results above argue for an important role of PAFR activation in mediating the phagocytosis of bacteria. The latter possibility is being actively investigated in our laboratory.

The CC chemokine MCP-1 appears to play an important role in the pulmonary anti-fungal response after *Aspergillus fumigatus* and *Cryptococcus neoformans* infection in mice (Huffnagel *et al.*, 1995; Blease *et al.*, 2001). Similarly, administration of MCP-1 prior to a systemic infection with *Pseudomonas aeruginosa* enhanced survival, an effect associated with enhanced bacterial phagocytosis and killing *in vitro* (Nakano *et al.*, 1994). Nevertheless, several studies have now shown that MCP-1 may shift the balance in favour of anti-inflammatory cytokine production after endotoxin challenge or during septic peritonitis (Matsukawa *et al.*, 2000; Zisman *et al.*, 1997; Hogaboam *et al.*, 1998). There was a marked increase in pulmonary MCP-1 concentrations 24 h after infection with *K. pneumoniae*. Pretreatment with UK-74,505 failed to affect MCP-1 concentrations significantly in our system, excluding a role for PAFR in modulating MCP-1 production.

In conclusion, our results suggest that PAF, by acting on its receptor, plays little role in the local production of chemokines and recruitment of leukocytes during *K. pneumoniae* infection in mice. However, the PAFR appears to contribute to the local production of TNF-α and to the ability of leukocytes to deal with the infecting bacteria. The latter roles of the PAFR may underlie the increased lethality observed in animals treated with a PAFR antagonist or in $PAFR^{-/-}$ mice. Thus, whereas PAFR antagonism appears to be an effective strategy to control the lung injury associated with a range of acute inflammatory stimuli, this receptor is also part of an effective innate immune response against bacterial infection in the lungs. The latter effects of PAFR antagonists may be relevant in humans and could limit any beneficial effects of the drugs in acute inflammatory conditions, such as sepsis.

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Platelet-activating factor receptor

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Abstract

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a biologically active phospholipid mediator. Although PAF was named for its potential to induce platelet aggregation, intense investigations have elucidated potent biological actions of PAF in a broad range of cell types and tissues. PAF acts by binding to a unique G-protein-coupled seven transmembrane receptor, and activates multiple intracellular signaling pathways. In the last decade, we have identified the PAF receptor structures, intracellular signaling mechanisms, and genomic organizations. Recently, we found a single nucleotide polymorphism of the human PAF receptor (A224D) with an allele frequency of 7.8% in Japanese. Cells expressing this receptor exhibited the reduced cellular signaling, although the binding parameters remain unchanged. We have established two different types of genetically altered mice, i.e. PAF receptor-overexpressing mouse and PAF receptor-deficient mouse. These mutant mice provide a novel and specific approach for identifying the pathophysiological and physiological functions of PAF in vivo. This review focuses on phenotypes of these mutant mice and summarizes the previous reports regarding PAF and PAF receptor.

Keywords: Platelet-activating factor; Phospholipid mediator; Intracellular signaling pathways

1. Introduction

Phospholipids are major components of cellular membranes. They are also known to be sources of arachidonic acid, which is metabolized into bioactive eicosanoids. Some

Abbreviations: PAF, platelet-activating factor; FISH, fluorescence in situ hybridization; PAFR-Tg, PAF receptor transgenic; PAFR-KO, PAF receptor-deficient; WT, wild-type; Mch, methacholine; TX, thromboxane; LT, leukotriene; BrdU, 5-bromo-2′-deoxyuridine

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3.1. Establishment of PAF receptor mutant mice

PAFR-Tg mice were created by injecting a transgene containing guinea-pig PAF receptor cDNA into the pronuclei of fertilized eggs from BDF1 female mice [47]. The genetic background of the mice used in the experiments was mostly a random mixture of C57BL/6 and DBA/2 alleles. The guinea-pig PAF receptor cDNA was placed under the regulation of cytomegalovirus-immediate early enhancer and β-actin promoter for ubiquitous expression in mice. However, Northern analysis revealed a varied pattern of transgene expression in the established transgenic mouse line: high levels of expression were seen in heart and skeletal muscle, medium levels in eye, skin, trachea and aorta, and barely detectable levels in neutrophils, brain, lung, liver, spleen, kidney, small intestine, uterus and testis. This may suggest that ubiquitous overexpression of PAF receptor was lethal to mouse embryos, so that the PAFR-Tg line that expressed PAF receptor in the pattern described above might have been naturally selected. Expression of the PAF receptor transgene in the early embryonic stage can be deduced from other studies using the same expression unit [48–50].

PAFR-KO mice were created by targeted gene disruption of the PAF receptor gene in E14-1 embryonic stem cells derived from 129/Ola mouse [51]. Unless otherwise stated, the genetic background of PAFR-KO mice is mostly a random mixture of 129/Ola and C57BL/6 alleles. Inactivation of the PAF receptor gene was confirmed by genomic Southern analysis and by Northern analysis of RNA isolated from neutrophils. Neither peritoneal macrophages nor lung tissues from PAFR-KO mice displayed any detectable binding sites for a PAF antagonist WEB 2086, which confirms the loss of surface receptors from these tissues. In PAFR-KO mice, a challenge with PAF no longer induced an increase in intracellular calcium concentrations in casein-elicited peritoneal exudate cells [51], a decrease in arterial pressure [51], a chemotactic response of microglia [52], or arrhythmogenic responses of ventricular myocytes [53]. These results provide compelling evidence that the targeted PAF receptor plays a crucial role in the PAF bioactions. No gross morphological abnormalities were detected in PAFR-KO mice.

3.2. Phenotypes of PAF receptor mutant mice

3.2.1. Reproduction

When heterozygous transgenic males were mated to wild-type (WT) females, the proportion of PAFR-Tg progeny to WT progeny (1:2.3) was significantly lower than that which would be expected from a Mendelian distribution (1:1) [47]. Despite the restricted pattern of transgene expression, a fraction of PAFR-Tg embryos still may not survive gestation. Heterozygous female PAFR-Tg mice produced fewer transgenic progenies, suggesting that a higher expression of PAF receptor in the maternal reproductive system easily causes disorders in fertilization. Interestingly, PAFR-KO mice possessed apparently normal reproductive potential [51]. Taken together, PAF is unlikely to be essential for murine reproduction in vivo, but spatiotemporally aberrant expression of the PAF receptor appears to prevent normal ontogeny by collapsing the regulated PAF receptor expression pattern in embryo and uterus. On the other hand, sperm from PAFR-KO mice, which have been backcrossed to C57BL/6 mice more than 10 times, gave a significantly lower rate of in vitro fertilization

(21.5%) than did WT sperm (66.7%) [54]. This report suggests that PAF potentiates the capacity of sperm for in vivo fertilization.

3.2.2. Bronchial hyperresponsiveness

Although there were no inflammatory symptoms in the airways of PAFR-Tg mice under physiological conditions, they showed congenital bronchial hyperresponsiveness to inhaled PAF and Mch, a muscarinic receptor agonist [47,55,56]. Because no significant hyperresponsiveness to serotonin was observed in PAFR-Tg mice [56], PAF receptor-overexpression is unlikely to affect general bronchial responsiveness. In response to PAF, the lung resistance in PAFR-Tg mice was increased by several folds largely due to bronchoconstriction, while the airways of WT mice were refractory to PAF [47]. The PAF-induced increase in lung resistance was greatly attenuated not only by the PAF antagonist WEB 2086, but also by a cyclooxygenase inhibitor indomethacin, a thromboxane (TX) synthase inhibitor ozagrel, a 5-lipoxygenase-activating protein inhibitor MK-886, and a cysteinyl leukotriene (LT) antagonist pranlukast [55]. An LTB_4 antagonist ONO-4057, however, had no effects. Therefore, TXA_2 and cysteinyl LTs, which are possibly released in response to PAF, may enhance the PAF-elicited airway response [57,58]. Since the inhibitory effect of each drug was fairly large, the direct role of PAF in bronchoconstriction seems to be much smaller than that of TXA_2 and cysteinyl LTs. Moreover, inhibition of TX synthesis seems to reduce the potent actions of cysteinyl LTs and vice versa. Probably, TXA_2 and cysteinyl LTs exert contractile effects synergistically on the airways of PAFR-Tg mice.

Pretreatment of PAFR-Tg mice with a PAF antagonist, a TX synthase inhibitor, or a cysteinyl LT antagonist significantly reduced the hyperresponsiveness to Mch, one of the major manifestations of asthma [47,55]. These results suggest that there is an interrelation between Mch and PAF hyperresponsiveness as well as a contribution of PAF to the etiology of asthma. However, the binding assays for muscarinic receptors revealed no difference in K_d or B_{max} value between PAFR-Tg and WT mice [56]. Thus, a potential mechanism underlying hyperresponsiveness to Mch is that Mch-stimulated release of PAF may lead to overproduction of TXA_2 and cysteinyl LTs in PAFR-Tg mice. Unexpectedly, Mch appears to mediate the bronchoconstrictive action of PAF in PAFR-Tg mice, since atropine, an antagonist for muscarinic receptors, strongly blocked the PAF-induced airway responses [56]. This report suggests that a synergistic effect between PAF and Mch also exists in the airways of PAFR-Tg mice.

3.2.3. Systemic anaphylaxis

Actively sensitized PAFR-KO mice showed drastically attenuated systemic anaphylactic responses—hypotension, heart rate change, lung resistance elevation with bronchoconstriction, and lung edema—after antigen challenge [51]. PAFR-KO mice were also more likely to survive systemic anaphylaxis compared with WT mice. These results demonstrate a dominant role of PAF in murine anaphylaxis, although there are various other anaphylactic mediators, such as eicosanoids, histamine, and serotonin.

3.2.4. Inflammation

When hydrochloric acid (HCl) was given intratracheally, PAFR-Tg mice showed more severe lung injury—increases in lung elastance, development of pulmonary edema, and

deterioration of gas exchange—compared to WT mice [59]. The symptoms of PAFR-KO mice were significantly milder than those of WT mice. But, the degree of neutrophil sequestration in the lung was similar in both mutant mice. These observations suggest that PAF is involved in the pathogenesis of acute lung injury caused by acid aspiration in mice, except for neutrophil sequestration. For induction of the lung injury, therefore, neutrophil sequestration is not sufficient, but PAF-activation of neutrophils (and/or an as yet unidentified pulmonary cell type) is probably required.

3.2.5. Endotoxic shock

In terms of sensitivity to LPS, phenotypes of PAFR-Tg and PAFR-KO mice were apparently conflicting. The lethality of LPS was significantly higher for PAFR-Tg mice than for WT mice [47]. WEB 2086 afforded good protection against LPS-induced death with PAFR-Tg mice. Contrary to expectations, PAFR-KO mice showed the same sensitivity to LPS as WT mice [51]; two major systemic responses, lethality and hypotension, were normal in PAFR-KO mice. In support of the data, peritoneal macrophages from WT and PAFR-KO mice produced equivalent levels of the two proinflammatory cytokines, IL-1β and IL-6, in response to LPS. PAF may, therefore, function not as an essential, but as an exaggerating factor for murine endotoxic shock. Since mice are less sensitive to LPS than other experimental animal species [60], the contribution of PAF to endotoxic shock might be congenitally low in mice. This might account for the normal sensitivity to LPS in PAFR-KO mice.

3.2.6. Brain

PAFR-KO mice had no apparent gross malformation in the brain [51], comparable to that seen with *LIS1* gene-disrupted mice [46]. This may suggest that Lis1 protein has other function(s) that are relevant to brain development but unrelated to PAF. PAFR-KO mice exhibited normal LTP and showed no obvious abnormality in excitatory synaptic transmission in the hippocampal CA1 region [61]. However, another group has shown the impairment of LTP in the dentate gyrus in PAFR-KO mice [62]. More detailed studies are needed to draw a definite conclusion with respect to physiological roles for PAF and the PAF receptor in central nervous system.

3.2.7. Skin

Abnormal skin pigmentation was observed in the ear and tail of PAFR-Tg mice, some of which spontaneously progressed into melanocytic tumors [47]. In the skin of the ear and tail, not only melanocytes but also keratinocytes proliferated highly. This hyperplasia increased with aging [63]. Of interest, these skin abnormalities had no effect on the coat color of PAFR-Tg mice, indicating a normal functioning of melanocytes in hair follicles. In an experiment using in situ hybridization, expression of the transgene (guinea-pig PAF receptor cDNA) was observed in keratinocytes but not in melanocytes [63]. Furthermore, the melanocytic tumors only weakly expressed guinea-pig PAF receptor mRNA and proteins (unpublished data). These results suggest that the hyperplasia of melanocytes is due to an indirect effect of PAF receptor overexpression. Since keratinocytes are known to produce growth factors for melanocytes, including basic fibroblast growth factor [64], endothelin-1 [65], and stem cell factor [66], PAF receptor-overexpression in keratinocytes

may result in abnormal production of various growth factors that can cause hyperplasia of melanocytes.

Accelerated proliferation of keratinocytes in PAFR-Tg mice was demonstrated by incorporation of 5-bromo-2′-deoxyuridine (BrdU) in vivo [63]. Treatment with a WEB 2086 ointment reduced the number of BrdU-positive cells. Hence, these results confirm the potential of PAF receptor-overexpression to proliferate keratinocytes in vivo. Interestingly, a decrease in BrdU-positive cells was also observed in WT mice, suggesting that PAF is involved in keratinocyte proliferation under physiological conditions.

PAFR-KO mice show no spontaneous abnormalities in the skin. Thus, PAF does not appear to be essential for the skin in terms of morphogenesis and proliferation under normal physiological conditions. It is noteworthy, however, that aberrant expression of the PAF receptor has the potential to elicit skin diseases.

3.2.8. PAF metabolism

As for low-density lipoprotein and thrombopoietin, their cognate receptors are reported to regulate the ligand concentration in the extracellular space [67,68]. Receptor-dependent metabolism of the ligands is likely to be responsible for the phenomenon. Thus, PAFR-KO mice, which have been backcrossed to C57BL/6 mice six to seven times, were used to examine the receptor-dependent metabolism, i.e. production and degradation of PAF. In casein-elicited peritoneal exudate cells rich in neutrophils, a calcium ionophore A23187 enhances PAF production [69]. PAF receptor deficiency, however, did not cause a change of either the basal PAF level or the PAF-producing ability of these cells. On the other hand, thioglycollate-elicited peritoneal macrophages from PAFR-KO mice exhibit half the capacity for PAF-degradation of WT mice [70]. The reduction was ascribed to the deficiencies of receptor-dependent PAF uptake and PAF acetylhydrolase release in PAFR-KO mice.

4. Closing remarks

This review has provided an update of our knowledge of PAF and the PAF receptor, focusing on the human PAF receptor mutant and the phenotypes of genetically altered mice.

In investigating the function of the PAF receptor, the overwhelming advantage of the transgenic approach over the conventional pharmacological one is the complete selectivity obtained in PAFR-Tg and PAFR-KO mice. Indeed, several PAF antagonists were demonstrated to have dual activities blocking PAF receptor-mediated activation but also inhibiting other enzymes, including cyclooxygenase [71], lipoxygenase [71], phospholipase A2 [72], acetylcholinesterase [73], and intracellular PAF acetylhydrolase [74,75]. Some other PAF antagonists inhibited histamine effects through interaction with the histamine G-protein-coupled receptor [76–78]. The disadvantage of the transgenic approach is the limitation of species; we are allowed to utilize only mouse, particularly in the case of a gene-targeted animal. Since much research on PAF has dealt with other species besides the mouse, e.g. human, dog, rat, and guinea-pig, it is conceivable that species-specific phenomena may cause confusion in interpreting the data. Historically, the use of new strategies and the interpretation of data derived from them become more sophisticated with time.

Although we recognize that PAF receptor mutant mice are not a panacea for PAF research, they should contribute greatly to our understanding of the physiological and pathophysiological functions of PAF, along with pharmacological and genetic approaches.

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