0022-3565/02/3031-45–51$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 303, No. 1
37382/1013557
JPET 303:45–51, 2002
Printed in U.S.A.
Copyright © 2002 by The American Society for Pharmacology and Experimental Therapeutics

Nonpeptide Antagonists of AT1 Receptor for Angiotensin II Delay the Onset of Acute Respiratory Distress Syndrome

SILVINA RAIDEN, KAREN NAHMOD, VÍCTOR NAHMOD, GUILLERMO SEMENIUK, YANINA PEREIRA, CLARISA ALVAREZ, MIRTA GIORDANO, and JORGE R. GEFFNER

Laboratory of Immunology, Institute of Hematologic Research, National Academy of Medicine (S.R., K.N., M.G., J.R.G.); Institute of Medical Research "Alfredo Lanari" (V.N., G.S., Y.P., C.A.), and Department of Microbiology, Buenos Aires University School of Medicine (M.G., J.R.G.), Buenos Aires, Argentina

Received April 12, 2002; accepted June 25, 2002

ABSTRACT

We have previously reported that losartan, a selective antagonist of AT1 receptors for angiotensin II (AII), strongly suppresses the activation of neutrophils by *N*-formylmethionyl-leucyl-phenylalanine (fMLP) through a mechanism that does not involve inhibition of AT1 receptors. Herein, we analyze whether losartan would prevent the development of the acute respiratory distress syndrome (ARDS) triggered by lung bacterial infection. We found that losartan (0.2–200 μg/kg/min) delays the onset of ARDS in Wistar rats challenged by i.t. instillation of *Bordetella bronchiseptica*. Although this effect was associated with a significant inhibition of lung-neutrophil recruitment, lung bacterial clearance was not impaired but rather, it was significantly improved. We also found that another nonpeptide AT1 receptor blocker, irbesartan, exerted similar effects to losartan, i.e., it was also able to inhibit neutrophil activation by fMLP and to delay the onset of ARDS in *B. bronchiseptica*-challenged rats. Neither the inhibitor of angiotensin-converting enzyme captopril, nor the nonselective peptide inhibitor of AII receptors saralasin reproduced these effects. Our data are consistent with the possibility that nonpeptide AT1 receptor blockers delay the onset of ARDS triggered by bacterial infection through a mechanism dependent, at least in part, on their ability to prevent neutrophil activation by *N*-formyl-peptides.

Angiotensin II (AII) is an important molecule controlling blood pressure and volume in the cardiovascular system. Most of the known effects of AII seem to be mediated via stimulation of the G protein-coupled AT1 receptor (Timmermans et al., 1993; Clauser et al., 1996). Losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(1*H*-tetrazol-5-yl biphenyl-4-yl) methyl] imidazole, potassium salt) is the prototype of the antagonists of AT1 receptor for angiotensin II. It was the first such drug available for clinical use since 1990 and actually it is widely used to manage systemic arterial hypertension (Johnston, 1995; Ardaillou, 1999; Timmermans, 1999).

We have previously reported that losartan impairs neutrophil activation triggered by *N*-formylmethionyl-leucyl-phenylalanine (fMLP) through a mechanism that does not involve blockade of AT1 receptors and depends, at least in part, on the inhibition of fMLP binding to neutrophil receptors for fMLP (FPRs) (Raiden et al., 1997, 2000). Taking this into account, and considering that bacteria induce neutrophil chemotaxis by releasing *N*-formyl peptides, we analyzed whether losartan was able to prevent lung-neutrophil recruitment in rats challenged by i.t. instillation of *Pseudomonas aeruginosa*. We found that losartan markedly decreases neutrophil accumulation in infected lungs (Raiden et al., 2000).

The acute respiratory distress syndrome (ARDS) is a devastating clinical syndrome of acute lung injury of high mortality rate (40–60%) despite intensive care using currently available drugs (Wyncoll and Evans, 1999; Ware and Matthay, 2000). It is the most severe form of a wide spectrum of pathological processes designated as acute lung injury. Lung injury in ARDS is caused by damage to the pulmonary vessels and alveoli mediated, at least in part, by activated neutrophils, resulting in massive pulmonary edema, neutrophil infiltration, and surfactant dysfunction (Weiland et al., 1986; Gattinoni et al., 1994; Wyncoll and Evans, 1999; Ware and Matthay, 2000).

In this work, we report that losartan delays the onset of ARDS in Wistar rats challenged by i.t. instillation of *Bordetella bronchiseptica*. Although this effect was associated with

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas, Agencia Nacional de Promoción Científica y Tecnológica, Universidad de Buenos Aires, Fundación "Roemmers", and Ministerio de Salud, Subsecretaría de Investigación y Tecnología, Argentina.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.037382.

ABBREVIATIONS: AII, angiotensin II; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; FPR, *N*-formylmethionyl-leucyl-phenylalanine receptor; ARDS, acute respiratory distress syndrome; IL, interleukin; ZAS, zymosan-activated serum; aIgG, human heat-aggregated IgG; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; AM, acetoxymethyl ester; CFU, colony-forming unit; TxA_2, thromboxane A_2.

a significant inhibition of lung-neutrophil recruitment, lung bacterial clearance was not impaired but rather, it was significantly improved. In addition, we found that another nonpeptide AT1 receptor blocker, irbesartan, exerted similar effects to those of losartan, i.e., it was also able to inhibit neutrophil activation by fMLP and to delay the onset of ARDS in *B. bronchiseptica*-challenged rats.

Materials and Methods

Reagents. Zymosan, fMLP, captopril, saralasin, and IL-8 were purchased from Sigma-Aldrich (St. Louis, MO). Zymosan-activated serum (ZAS), used as a source of C5a, was prepared by incubating 15 mg of zymosan with 1 ml of fresh serum with end-over-end rotation for 1 h at 37°C. Then serum was heat-inactivated for 30 min at 56°C. After spin at 1000*g* for 15 min at 4°C, the supernatant was collected and stored at −70°C. Human heat-aggregated IgG (aIgG) was prepared by heating human IgG at a concentration of 5 mg/ml for 12 min at 63°C. Then aIgG was centrifuged at 10,000*g* for 5 min, and the precipitate was discarded.

Preparation of Neutrophils. Citrated blood samples were obtained from adult male Wistar rats, and neutrophils were isolated by dextran sedimentation and Histopaque gradient centrifugation, as described previously (Reinhardt et al., 1997). Contaminant erythrocytes were removed by hypotonic lysis. After washing, the cells (>86% neutrophils on May Grunwald-Giemsa-stained Cytopreps) were resuspended at the desired concentration in RPMI 1640 medium (Invitrogen, Carlsbad, CA) and supplemented with 1% heat-inactivated fetal calf serum (Invitrogen).

Measurement of Fluctuations in Intracellular Ca^{2+} Concentrations $[Ca^{2+}]_i$. Changes in $[Ca^{2+}]_i$ were monitored using fluo-3/AM, as described previously (Kao et al., 1989). Briefly, neutrophils, suspended at a concentration of 5×10^6 cells/ml in RPMI 1640 medium were incubated with 4 μM fluo-3/AM for 30 min at 30°C. Then loaded cells were washed twice and resuspended at 5×10^6 cells/ml in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum. Aliquots of 50 μl of this cell suspension were then added to 450 μl of RPMI 1640 medium containing 5% fetal calf serum and warmed at 37°C. The samples were immediately loaded onto the flow cytometer, and the basal fluorescence (FL1) was recorded during 15 s. Then cells were activated by different stimuli, in the absence or presence of AT1 inhibitors, and the fluorescence was recorded during an additional 100 s. Acquisition of samples was performed at 37°C. Fluctuations in cytoplasmic free calcium concentrations were recognized as alterations in fluo-3 fluorescence intensity over time. Data were analyzed by using CellQuest software (BD Biosciences, Mountain View, CA). A gate based on forward and side scatters was used to exclude debris. To determine the percentage of cells responding to the stimuli, several nonoverlapping 10-s-wide time gates were used to create one-parameter histograms of the logarithmic fluo-3/AM intensity. A control histogram was created during the first 10 s of acquisition before the addition of the stimulus. Histograms from gates corresponding to 10 to 20 s after the addition of each stimulus were compared with the control histogram to determine the percentage of cells demonstrating increased fluorescence. This percentage corresponds to the proportion of cells that responds with Ca^{2+} flux to stimulation.

Assessment of Lung Myeloperoxidase Activity. Neutrophil infiltration into the lung was quantified by measuring myeloperoxidase activity in lungs 7 h after challenging (Shanley et al., 1997). Briefly, lungs were homogenized and treated with Triton X-100, in potassium phosphate buffer, pH 6.0. After centrifugation at 2000*g* for 30 min, the supernatant fluids were reacted with H_2O_2 (30% stock diluted 1:100; Sigma-Aldrich) in the presence of *O*-dianisidine hydrochloride (1 mg/ml) (Sigma-Aldrich), and the myeloperoxidase content was reported as change in optical density at 460 nm.

Histopathological Studies. Rat lung tissue was fixed with 10% buffered formalin, pH 7.2, dehydrated in graded alcohols, embedded in paraffin, and cut into 6-μm sections. Mounted sections were stained for light microscopy with hematoxylin and eosin. Sections were examined for features of lung injury, including congestion, alveolar edema, and accumulation of inflammatory cells. All morphological studies were done by a pathologist blinded with respect to the different experimental groups studied.

Bacteriological Cultures of Lung Homogenates. Rats were challenged by i.t. instillation of live *B. bronchiseptica* [50 μl, 10^9 colony-forming units (CFU)/ml]. Immediately thereafter, losartan (20 μg/kg/min) or saline (control) was administered by continuous i.v. infusion. Animals were sacrificed 7 h after challenging, the lungs were exposed aseptically, removed, and homogenized. The concentration of bacteria was quantified by placing successive 10-fold dilutions of the suspension on tryptone soy agar plates and scoring visible colonies after 24 h of incubation at 37°C. Results were expressed as CFU per lung.

Animal Models. Adult male Wistar rats weighing about 250 g were used in all experiments. Animals were housed under standard light (lights on from 6:00 AM to 6:00 PM) and temperature (23°C) conditions. Food and water were available ad libitum. Rats were anesthetized i.p. with urethane (1.2 g/kg of body weight), and the trachea was exposed. Then 50 μl of a *B. bronchispetica* suspension (10^9 CFU/ml) was instillated via an intratracheal catheter during inspiration. Immediately thereafter, losartan (0.2–200 μg/kg/min) or saline (control) was administered by continuous i.v. infusion through the jugular vein. Losartan infusion was maintained throughout the experiment. Rats were allowed to breath spontaneously during the experiment without oxygen therapy. The measurements of PaO_2, $PaCO_2$, and pH were then made at different times to assess the extent of respiratory failure, using a 280 blood gas analyzer (Ciba-Corning Co., Tarrytown, NY), and a sample volume of 0.3 ml from carotid artery. Acute lung injury was considered to be present if PaO_2/FiO_2 was ≤300 and ARDS if PaO_2/FiO_2 ≤200 (FiO_2 = 0.21).

Statistical Analysis. Results are expressed as means ± S.E.M. Statistical significance was determined using Student's *t* test. A probability level of $p < 0.05$ was considered statistically significant.

Results

Losartan Prevents the Drop in PaO_2/FiO_2 Values Triggered by Instillation of *B. bronchiseptica*. Instillation (i.t.) of *B. bronchiseptica* in adult Wistar rats induced deterioration of gas exchange, which was not observed in the saline-treated group (Fig. 1). Acute lung injury ($PaO_2/FiO_2 \leq 300$) and ARDS ($Pa\ O_2/FiO_2 \leq 200$) were observed at 5 and 7 h after challenging, respectively. Sixteen rats were originally instillated with *B. bronchiseptica*, but two animals died before 7 h and, consequently, were excluded from analysis.

We next examined whether losartan could prevent lung injury triggered by instillation of *B. bronchiseptica*. The results obtained are shown in Fig. 2. Losartan (0.2–200 μg/kg/min) did not modify PaO_2/FiO_2 ratio in saline-instillated rats, whereas it markedly prevented the decrease in PaO_2/FiO_2 values triggered by instillation of *B. bronchiseptica*. Significant effects were observed at doses of 2 to 200 μg/kg/min. Because there were no differences between doses of 20 and 200 μg/kg/min, subsequent experiments were performed using 20 μg/kg/min losartan.

Having shown that losartan prevented the decrease in PaO_2/FiO_2 values, we sought to analyze its effect on pCO_2, pH, and the recruitment of neutrophils in the lung, measured as the increase in lung myeloperoxidase content. Table 1 shows that losartan prevented the decrease in pH values, the

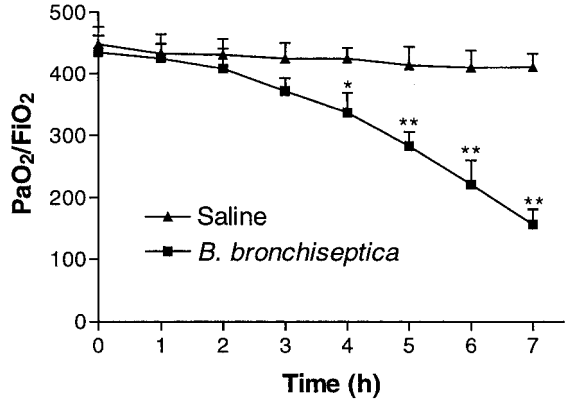

Fig. 1. Time course study of the development of ARDS in Wistar rats challenged by i.t. instillation of *B. bronchiseptica*. Measurements of PaO_2 were performed at different times after i.t. instillation of saline (50 μl, $n = 6$) or *B. bronchiseptica* (50 μl, 10^9 CFU/ml, $n = 14$). Data are expressed as PaO_2/FiO_2 values ($FiO_2 = 0.21$) and represent the arithmetic mean ± S.E.M. Statistical significance: *, $p < 0.05$ and **, $p < 0.01$, compared with saline-treated rats.

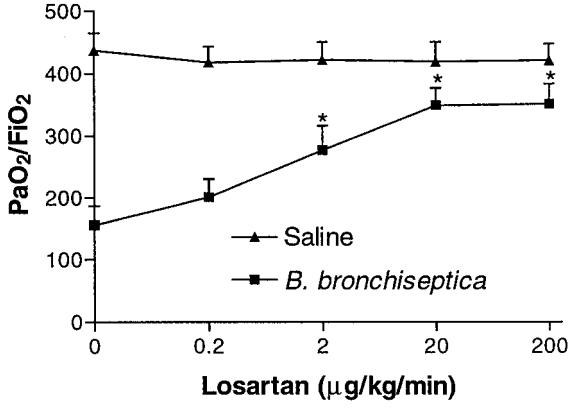

Fig. 2. Losartan prevents the decrease in PaO_2/FiO_2 values in rats challenged by *B. bronchiseptica*. Measurements of PaO_2 were performed at 7 h after i.t. instillation of saline (50 μl, $n = 4–6$) or *B. bronchiseptica* (50 μl, 10^9 CFU/ml, $n = 6–7$). Rats were treated with saline or losartan (0.2, 2, 20, and 200 μg/kg/min), which was administered by continuous i.v. infusion through the jugular vein. Data are expressed as PaO_2/FiO_2 values ($FiO_2 = 0.21$) and represent the arithmetic mean ± S.E.M. Statistical significance: *, $p < 0.01$, compared with rats challenged by *B. bronchiseptica* and treated with saline.

increase in pCO_2 as well as significantly inhibited lung neutrophil influx.

The typical histopathology of the lungs from rats challenged by *B. bronchiseptica* is depicted in Fig. 3, which shows thickened alveolar septae and a marked increase in cellularity dominated by polymorphonuclear leukocytes. As expected, these signs of inflammation were much less evident in losartan-treated rats.

We then performed additional experiments to establish whether losartan could prevent the progressive deterioration of gas exchange when given 4 h after the instillation of *B. bronchiseptica*, time at which a significant decrease in PaO_2/FiO_2 values was observed (Fig. 1). As shown in Fig. 4, there was a much more slow decrease in PaO_2/FiO_2 values in rats treated with losartan than in those treated with saline.

Losartan Improves Survival of Rats Challenged with *B. bronchiseptica*. Fig. 5 shows that losartan delayed mortality of infected rats. In fact, at 10 h after instillation of *B. bronchiseptica* none of saline-treated animals were alive, whereas none of the 10 animals treated with losartan died at this time point. However, at 18 h after challenging, there was only one survivor in the losartan-treated group. As observed for untreated rats challenged with *B. bronchiseptica*, the death of losartan (20 μg/kg/min)-treated rats was preceded by an increase in lung-neutrophil recruitment. Thus, myeloperoxidase content, measured as absorbance change at 460 nm, increased from 0.58 ± 0.22 at 7 h to 0.96 ± 0.24 at 11 h after challenging ($n = 5$, $p < 0.05$). Moreover, a progressive deterioration of gas exchange was also observed: $PaO_2/FiO_2 = 337 \pm 28$ versus 225 ± 33, mean ± S.E.M. ($n = 6$) for losartan (20 μg/kg/min)-treated rats at 7 and 11 h after instillation of *B. bronchiseptica*.

Losartan Does Not Impair but Rather It Improves Lung Bacterial Clearance. We then analyzed the effect of losartan treatment on the bacterial burden. Quantitative bacteriology was performed on lung homogenates from rats killed 7 h after instillation of *B. bronchispetica*. Surprisingly, we observed that the amount of *B. bronchiseptica* recovered from the lungs was not higher, but rather it was significantly lower in the losartan-treated group (mean CFU/lung = $2.4 \pm 0.8 \times 10^7$, $n = 6$) compared with the saline-treated group ($8.1 \pm 1.2 \times 10^7$, $n = 6$, $p < 0.05$). This unexpected finding led as to analyze whether losartan would be able to exert a bacteriostatic or bactericidal effect. Experiments were performed by culturing *B. bronchiseptica* in tryptic soy broth for 24 h at 37°C. We observed no differences in the number of bacteria harvested from cultures performed in the absence or presence of losartan (1–200 μg/ml) (data not shown).

Effect of Other Antagonists of the Renin-Angiotensin System on Development of ARDS Triggered by Instillation of *B. bronchiseptica*. We next analyze whether other antagonists of the renin-angiotensin system were also able to delay the onset of ARDS in infected rats. To this aim, we used captopril, an inhibitor of the angiotensin-converting enzyme, and saralasin, a nonselective peptide inhibitor of AII receptors (Regoli et al., 1974; Timmermans et al., 1993; Clauser et al., 1996). None of these compounds were able to prevent the drop in PaO_2/FiO_2 values in rats challenged by *B. Bronchiseptica* (Fig. 6). In contrast, it was markedly prevented by irbesartan, a nonpeptide blocker of AT1 receptors,

TABLE 1

Blood gas variables and lung neutrophil influx in *B. bronchiseptica*-challenged rats treated with losartan

The determinations of PaO_2/FiO_2, $PaCO_2$, pH, and lung myeloperoxidase (MPO) were performed at 7 h after i.t. instillation of saline (50 μl) or *B. bronchiseptica* (50 μl, 10^9 CFU/ml). Data represent the arithmetic mean ± S.E.M. ($n = 6–8$).

Group	PaO_2/FiO_2	$PaCO_2$ (mm Hg)	pH	Lung MPO (Abs 460 nm)
Saline i.t. + Saline i.v. (Controls)	448 ± 29	38 ± 5	7.38 ± 0.05	0.32 ± 0.13
B. bronchiseptica i.t. + Saline i.v.	151 ± 29*	59 ± 6*	7.19 ± 0.04*	1.37 ± 0.3*
B. bronchiseptica i.t. + Losartan i.v. (20 μg/kg/min)	345 ± 19**	42 ± 4**	7.31 ± 0.02**	0.69 ± 0.23**

Statistical significance: * $p < 0.01$ compared with controls; ** $p < 0.01$ compared with rats challenged by *B. bronchiseptica* and treated with saline.

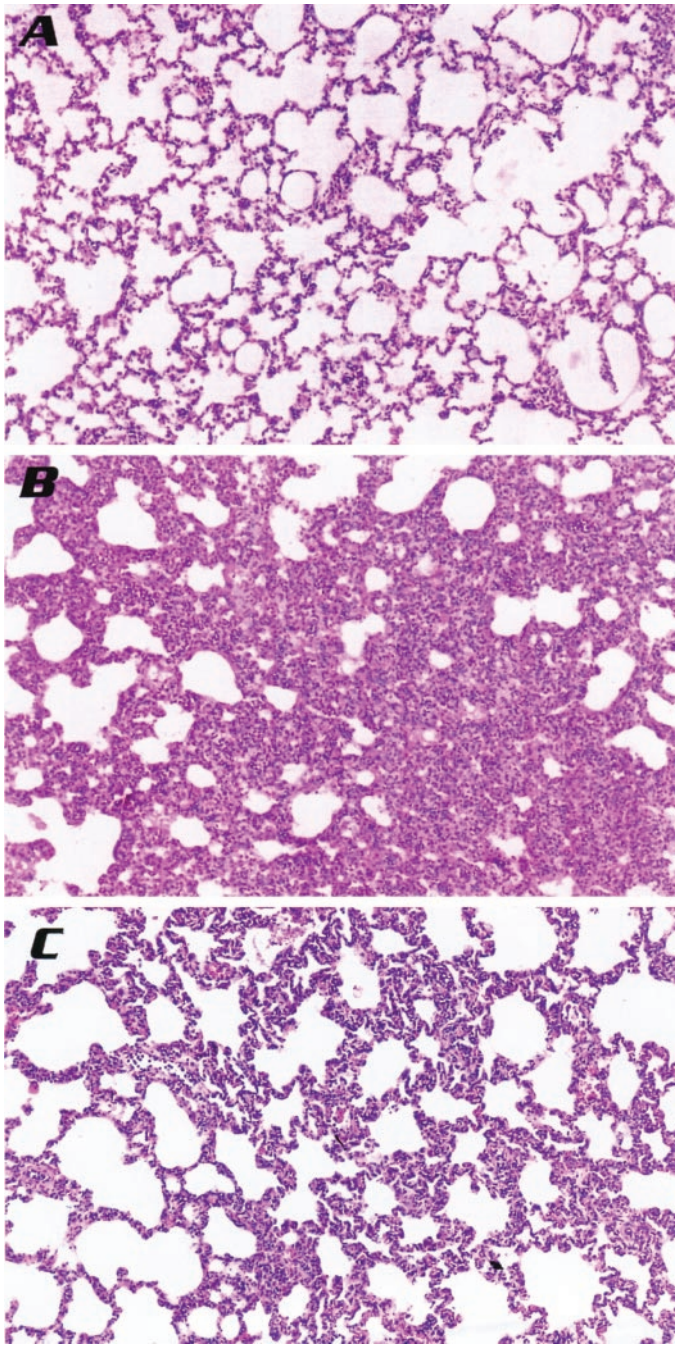

Fig. 3. Histological examination of lung sections. Seven hours after i.t. instillation of saline (50 μl) or *B. bronchiseptica* (50 μl, 10^9 CFU/ml), lung sections were obtained from saline-instillated rats treated with saline (a), *B. bronchiseptica*-instillated rats treated with saline (b), and *B. bronchiseptica*-instillated rats treated with losartan (20 μg/kg/min) (c). Sections were stained for light microscopy with hematoxylin and eosin.

based on modifications of losartan's prototypic chemical structure (Timmermans, 1999).

Nonpeptide AT1 Receptor Blockers Irbesartan, Candesartan, and Valsartan Share with Losartan the Ability to Selectively Inhibit Neutrophil Activation Triggered by fMLP. Taking into account that irbesartan prevented lung injury in a similar manner to losartan, we next examined whether it was also able to inhibit neutrophil activation by fMLP. Studies were performed in isolated neutrophils by measuring rises in intracellular

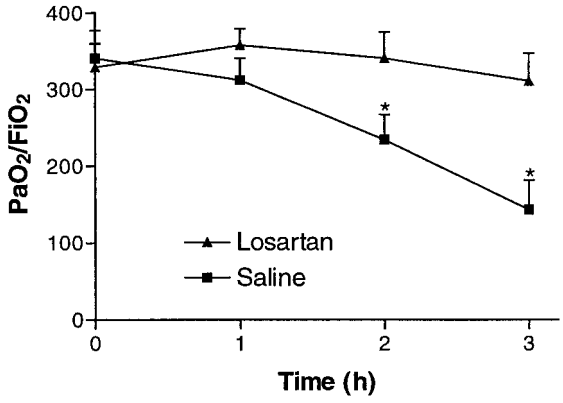

Fig. 4. Losartan delays the decrease in PaO_2/FiO_2 values in rats challenged by *B. bronchiseptica* even when it was administered after the onset of inflammation. Rats were i.t. instillated with *B. bronchiseptica* (50 μl, 10^9 CFU/ml). After 4 h ($t = 0$ in the figure), they were treated with saline or losartan (20 μg/kg/min), which was administered by continuous i.v. infusion, and PaO_2 was then determined at different times. Data are expressed as PaO_2/FiO_2 values ($FiO_2 = 0.21$) and represent the arithmetic mean ± S.E.M. of five to seven animals. Statistical significance: *, $p < 0.05$, compared with rats challenged by *B. bronchiseptica* and treated with saline.

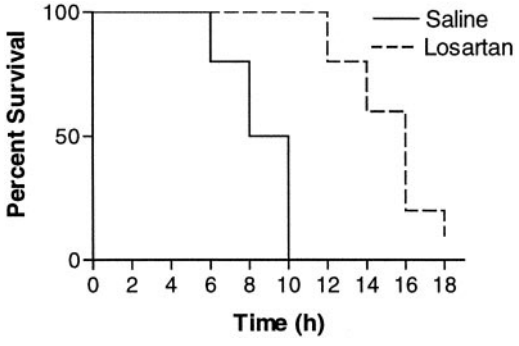

Fig. 5. Losartan improves survival of *B. bronchiseptica*-challenged rats. Animal survival was analyzed at different times after instillation of *B. bronchiseptica* (50 μl, 10^9 CFU/ml) in rats treated with saline or losartan (20 μg/kg/min). Data are expressed as percentage of survival ($n = 10$ for each group).

Ca^{2+} concentrations triggered by fMLP. Irbesartan almost completely inhibited Ca^{2+} transients triggered by fMLP without affecting those responses triggered by other stimuli such as ZAS, aIgG (Fig. 7) or IL-8 (data not shown). Interestingly, similar inhibitory effects were observed using two additional nonpeptide blockers of AT1 receptors (5, 16), candesartan and valsartan (10 μg/ml), as indicated by the percentage of cells activated by fMLP of 75 ± 14, 16 ± 5, and 7 ± 4% (control, candesartan, and valsartan, respectively; mean ± S.E.M., $n = 5$, $p < 0.001$, candesartan and valsartan versus controls). In agreement with the observations made with losartan and irbesartan, Ca^{2+} transients triggered by aIgG, ZAS, or IL-8 were unmodified by candesartan and valsartan (data not shown).

Discussion

We have previously reported that losartan, a nonpeptide blocker of AT1 receptors for AII, inhibits neutrophil activation triggered by fMLP (Raiden et al., 1997). Studies performed in vitro showed that neutrophil responses triggered by fMLP such as adherence, shape change, and the production of oxygen-reactive intermediates were markedly sup-

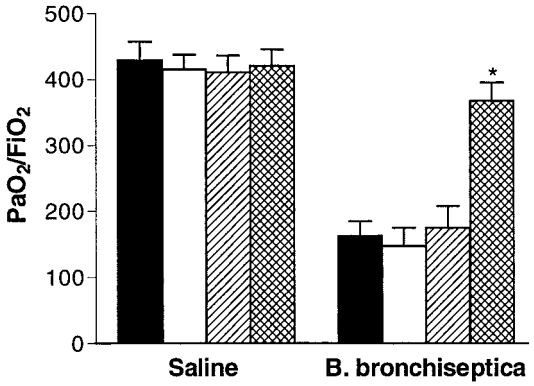

Fig. 6. Irbesartan, but not captopril nor saralasin, prevents the decrease in PaO_2/FiO_2 values in rats challenged by *B. bronchiseptica*. Measurements of PaO_2 were performed at 7 h after i.t. instillation of saline (50 μl, n = 3–5) or *B. bronchiseptica* (50 μl, 10^9 CFU/ml, n = 5). Rats were treated with saline (■), captopril 50 μg/kg/min (□), saralasin 50 μg/kg/min (▨), and irbesartan 20 μg/kg/min (▩), which were administered by continuous i.v. infusion through the jugular vein. Data are expressed as PaO_2/FiO_2 values (FiO_2 = 0.21) and represent the arithmetic mean ± S.E.M. Statistical significance: *, $p < 0.01$, compared with rats challenged by *B. bronchiseptica* and treated with saline.

pressed by losartan, whereas the responses triggered by other stimuli such as immune complexes, lectins, zymosan, and C5a were unmodified (Raiden et al., 1997). Studies performed in Wistar rats, on the other hand, indicated that losartan prevented lung-neutrophil recruitment triggered by i.t. instillation of fMLP, without affecting neutrophil accumulation induced by immune complexes, zymosan, and C5a (Raiden et al., 2000). The ability of losartan to antagonize fMLP-mediated responses does not involve inhibition of AT1 receptors and could be explained, at least in part, by its capacity to inhibit the binding of fMLP to FPR (Raiden et al., 1997, 2000). Interestingly, both AT1 and FPR belong to the class of G protein-coupled seven-transmembrane domain receptors (Murphy, 1994; Timmermans et al., 1997) and share 31% sequence identity (Bernstein and Alexander, 1992). Because the ligand binding sites on these receptors are not well defined, we cannot determine the degree of homology in their binding pockets. Based on studies using chimeric receptors, site-directed mutagenesis, and inhibition assays using FPR-derived peptides, possible domains for fMLP binding have been identified in extracellular loops and all transmembrane segments (Perez et al., 1993, 1994; Quehenberger et al., 1993). In a more recent study, Miettinen et al. (1997), using site-directed mutagenesis, identified 10 putative transmembrane amino acids that may participate in binding of formylated peptides, which are located in the second, third, fourth, fifth, sixth, and seventh transmembrane domains. Regarding AT1 receptor, it also seems that the binding of both angiotensin II and nonpeptide antagonists that block angiotensin binding to this receptor (i.e., losartan) is dependent on several interactions that involve different receptor domains (Feng et al., 1995; Noda et al., 1995; Hunyady et al., 1998). Interestingly, Hoe and Saavedra (2002) have recently shown that the most important amino acid for losartan binding to AT1 receptor is a valine located in position 108, which is conserved in FPR. This position corresponds to the third transmembrane domain, which has 32% identity in both FPR and AT1 receptor. Another amino acid that seems to be involved in the binding of nonpeptide antagonists to AT1 receptor is an asparagine residue located in position 294 (Hunyady et al., 1998), also conserved in FPR, in a region (291–299) in which seven of the nine residues are identical in both FPR and AT1 receptor. Further studies are required to determine whether residues 108 and 294 are involved in the binding of losartan to FPR.

The experiments described in this report were performed to test the hypothesis that losartan would exert a protective effect in an animal model of ARDS. This hypothesis is based on our recent findings showing that losartan improves survival of *P. aeruginosa*-infected rats (Raiden et al., 2000). In the present work, we report that losartan delays the onset of ARDS in Wistar rats challenged by i.t. instillation of *B. bronchiseptica*. We also show that another nonpeptide blocker of AT1 receptors, irbesartan, exerts similar effects to those of losartan; it is also able to selectively inhibit neutrophil activation by fMLP, as well as to delay the onset of ARDS in infected rats.

Increasing evidence indicates that phagocytic cells express AT1 receptors, and that activation of these receptors by angiotensin II triggers a variety of inflammatory responses such as cytosolic calcium changes (Lijnen et al., 1997), activation of nuclear factor-κB (Kranzhofer et al., 1999), and production of tumor necrosis factor-α in mononuclear phagocytes (Nahmod et al., 1992), as well as neutrophil chemotaxis (Elferink and de Koster, 1997). However, although local production of AII increases at least 5-fold during the course of acute lung injury induced by *B. bronchiseptica* (S. Raiden, unpublished data), the mechanisms through which nonpeptide blockers of AT1 receptors delay the onset of ARDS do not seem to involve the inhibition of AT1 receptors, because neither captopril, an ACE inhibitor, nor saralasin, a nonselective inhibitor of AII receptors (Regoli et al., 1974; Timmermans et al., 1993; Clauser et al., 1996), was able to prevent the drop in PaO_2/FiO_2 values in infected rats. Our results are consistent with the possibility that losartan delays the onset of ARDS triggered by lung-bacterial infection by virtue of its ability to antagonize FPR, decreasing neutrophil accumulation in infected lungs.

In a study directed to analyze the role of formyl peptide (pathogen-derived) and chemokine (host-derived) chemoattractants in lung-leukocyte recruitment, in a mouse model of pneumococcal pneumonia, Fillion et al. (2001) found that both types of chemoattractants contribute to the recruitment of either neutrophils or mononuclear phagocytes. In regard to neutrophils, it was found that treatment of mice with a formyl peptide receptor antagonist (Boc-PLPLP) resulted in 40% reduction in neutrophil counts recovered in bronchoalveolar lavage fluid, compared with infected mice receiving placebo. Interestingly, despite the differences between experimental models used in Fillion's study and in our work, we found a similar inhibition in neutrophil recruitment to infected-lungs, as a consequence of losartan-treatment (Table 1). However, it is important to note that even though losartan was continuously administered to infected rats, its ability to prevent lung neutrophil recruitment declined 7 h after challenging, supporting a change in the mechanisms responsible for neutrophil accumulation in the lungs from a fMLP-dependent to a fMLP-independent pathway. This change could be related to the local production of C-X-C chemokines, which may act as potent chemoattractants for neutrophils. In fact, in the model of pneumococcal pneumonia described by Fillion

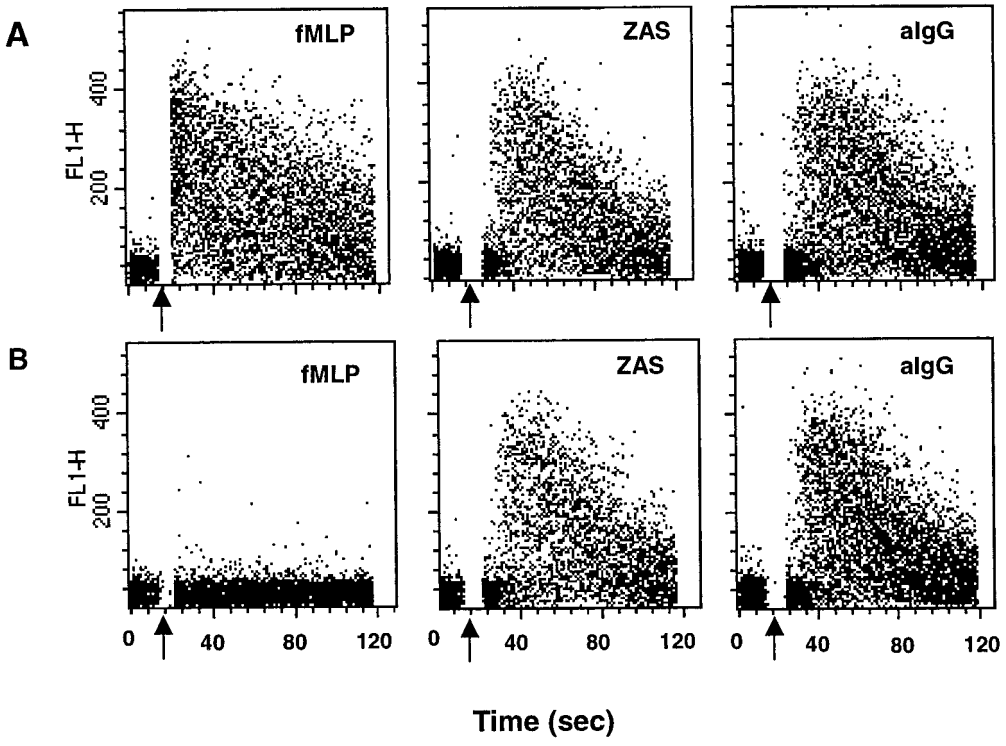

Fig. 7. Nonpeptide blockers of AT1 receptors selectively inhibits the rise in intracellular Ca^{2+} concentrations triggered by fMLP. Rat neutrophils were loaded with fluo-3/AM as described under *Materials and Methods*. The basal level of fluo-3 fluorescence was recorded for about 15 s. Then fMLP (10^{-8} M), ZAS (1/10) or aIgG (500 μg/ml) was added in the absence (A) or presence of irbesartan (10 μg/ml) (B), and fluorescence was recorded for 100 s. The increase in $[Ca^{2+}]_i$ was recognized as an increase in fluo-3 fluorescence. Results from a representative experiment (n = 4–5) are depicted.

et al. (2001), the production of MIP-2 and KC, two C-X-C chemokines, peaked in the lung at 4 h, and seemed to plateau from 8 to 24 h after the infection.

The mechanisms through which losartan delay the onset of ARDS could also involve additional pathways unrelated to its ability to antagonize fMLP-triggered responses. A number of reports have shown that nonpeptide AT1 receptor blockers such as losartan, its active metabolite EXP3174, and irbesartan, are also able to competitively block the thromboxane A_2 (TxA_2)/prostaglandin endoperoxide H_2 receptor (Liu et al., 1992; Bertolino et al., 1994; Li et al., 1997, 1998; Li et al., 2000). These results should be taken into account because thromboxane A_2 seems to be involved in the development of acute lung injury and ARDS. Experiments performed ex vivo, in rabbit heart-lung preparations, showed that TxA_2 receptor blockade ameliorates acute lung injury triggered by oleic acid (Thies et al., 1996; Goff et al., 1997). Studies carried out in a sheep model of ARDS showed that TxA_2 receptor blockade successfully blunted the early pulmonar hypertension seen after endotoxin administration but did not affect the subsequent increase in pulmonary capillary permeability (Wisner et al., 1988). Moreover, three clinical studies have suggested that ketoconazole, an inhibitor of thromboxane synthase, may be effective in preventing the development of ARDS in high-risk critically ill patients (Slotman et al., 1988; Yu and Tomasa, 1993; Sinuff et al., 1999). In contrast, a recent large multicenter trial did not confirm these promising initial reports and found no improvement in survival, ventilator-free days, organ failure-free days, or any measure of lung function, in ketoconazole-treated patients (ARDS Network Authors, 2000). Further studies are required to define whether the antagonistic actions of losartan and irbesartan on TxA_2 receptors play any role in the delay of the onset of ARDS observed in our experimental model.

We were surprised to find that pulmonary clearance of bacteria was augmented in losartan-treated rats, despite the reduced recruitment of neutrophils in the lung. Two hypotheses should be considered to explain this unexpected result. First, inflammatory response in ARDS seems to be excessive relative to the burden of bacterial infection (Weiland et al., 1986; Gattinoni et al., 1994; Wyncoll and Evans, 1999; Ware and Matthay, 2000). Although the phagocytic capacity afforded by neutrophil influx into the lung in response to bacteria is essential to defense capabilities against invading bacteria, an excessive inflammatory reaction causes a high degree of tissue destruction that may result in the impairment of bacterial clearance (Doring and Dauner, 1988; Chmiel et al., 1999). Thus, the possibility exists that losartan may improve bacterial clearance by virtue of its ability to transiently protect against tissue injury after intrapulmonary deposition of bacteria. Alternatively, the possibility exists that the improvement of bacterial clearance induced by losartan may be related to an action exerted on alveolar macrophages, cells that play a critical role in host defense mechanisms against lung infection (Broug-Holub et al., 1997; Kooguchi et al., 1998; Zhang et al., 2000). Although we have no direct evidence in vivo supporting this possibility, we have recently found that culture of rat peritoneal macrophages with losartan increases the ability of macrophages to ingest bacteria (S. Raiden, unpublished data).

We demonstrate herein for the first time that nonpeptide blockers of AT1 receptors for AII delay the onset of ARDS triggered by bacterial infection. However, it should be pointed out that even though dramatic improvement was observed at early time points, there was no long-term protection. Our results support the notion that AT1 receptor antagonists might prove to be useful tools to delineate early and late mechanisms of ARDS.

Acknowledgments

We thank Selma Tolosa and Nelly Villagra for technical assistance and Maria Rita Furnkorn for secretarial assistance.

References

Ardaillou R (1999) Angiotensin II receptors. *J Am Soc Nephrol* **10 (Suppl 11):** S30–S39.

ARDS Network Authors (2000) Ketoconazole for early treatment of acute lung injury and acute respiratory distress syndrome. *J Am Med Assoc* **283:**1995–2002.

Bernstein KE and Alexander RW (1992) Counterpoint: molecular analysis of the angiotensin II receptor. *Endocr Rev* **13:**381–386.

Bertolino F, Valentin JP, Maffre M, Jover B, Bessac AM, and John GW (1994) Prevention of thromboxane A2 receptor-mediated pulmonary hypertension by a nonpeptide angiotensin II type 1 receptor antagonist. *J Pharmacol Exp Ther* **268:**747–752.

Broug-Holub E, Toews GB, van Iwaarden JF, Strieter RM, Kunkel SL, Paine R, and Standiford TJ (1997) Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumonia*: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. *Infect Immun* **65:**1139–1146.

Chmiel JF, Konstan MW, Knesebeck JE, Hilliard JB, Bonfield TL, Dawson DV, and Berger M (1999) IL-10 attenuates excesive inflammation in chronic *Pseudomonas* infection in mice. *Am J Resp Crit Care Med* **160:**2040–2047.

Clauser E, Curnow KM, Davies E, Conchon S, Teutsch B, Vianello B, Monnot C, and Corvol P (1996) Angiotensin II receptors: protein and gene structures, expression and potential pathological involvement. *Eur J Endocrinol* **134:**403–411.

Doring G and Dauner HM (1988) Clearance of *Pseudomonas aeruginosa* in different rat lung models. *Am Rev Respir Dis* **138:**1249–1253.

Elferink JG and de Koster BM (1997) The stimulation of human neutrophil migration by angiotensin II: its dependence on Ca^{2+} and the involvement of cyclic GMP. *Br J Pharmacol* **121:**643–648.

Feng YH, Noda K, Saad Y, Liu XP, Husain A, and Karnik SS (1995) The docking of Arg2 of angiotensin II with Asp281 of AT1 receptor is essential for full agonism. *J Biol Chem* **270:**12846–12850.

Fillion I, Ouellet N, Simard M, Bergeron Y, Sato S, and Bergeron MG (2001) Role of chemokines and formyl peptides in pneumococcal pneumonia-induced monocyte/macrophage recruitment. *J Immunol* **166:**7353–7361.

Gattinoni L, Bombino M, and Pelosi P (1994) Lung structure and function in different stages of ARDS. *J Am Med Assoc* **271:**1772–1779.

Goff CD, Corbin RS, Theiss SD, Frierson HF, Cephas GA, Tribble CG, Kron IL, and Young JS (1997) Postinjury thromboxane receptor blockade ameliorates acute lung injury. *Ann Thorac Surg* **64:**826–829.

Hoe KL and Saavedra JM (2002) Site-directed mutagenesis of the gerbil and human angiotensin II AT(1) receptors identifies amino acid residues attributable to the binding affinity for the nonpeptidic antagonist losartan. *Mol Pharmacol* **61:**1404–1415.

Hunyady L, Ji H, Jagadeesh G, Zhang M, Gaborik Z, Mihalik B, and Catt KJ (1998) Dependence of AT1 angiotensin receptor function on adjacent asparagines residues in the seventh transmembrane helix. *Mol Pharmacol* **54:**427–434.

Johnston CI (1995) Angiotensin receptor antagonists: focus on losartan. *Lancet* **346:**1403–1407.

Kao JPY, Harootunian AT, and Tsien RY (1989) Photochemically generated cytosolic calcium pulses and their detection by fluo-3. *J Biol Chem* **264:**8179–8184.

Kooguchi K, Hashimoto S, Kobayashi A, Kitamura Y, Kudoh I, Wiener-Kronish J, and Sawa T (1998) Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. *Infect Immun* **66:**3164–3169.

Kranzhofer R, Browatzki M, Schmidt J, and Kubler W (1999) Angiotensin II activates the proinflammatory transcription factor nuclear factor-κB in human monocytes. *Biochem Biophys Res Commun* **257:**826–828.

Li P, Ferrario CM, and Brosnihan KB (1997) Nonpeptide angiotensin II antagonist losartan inhibits thromboxane A2-induced contractions in canine coronary arteries. *J Pharmacol Exp Ther* **281:**1065–1070.

Li P, Ferrario CM, and Brosnihan KB (1998) Losartan inhibits thromboxane A2-induced platelet aggregation and vascular constriction in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **32:**198–205.

Li P, Fukuhara M, Diz DI, Ferrario CM, and Brosnihan KB (2000) Novel angiotensin II AT(1) receptor antagonist irbesartan prevents thromboxane A(2)-induced vasoconstriction in canine coronary arteries and human platelet aggregation. *J Pharmacol Exp Ther* **292:**238–246.

Lijnen P, Fagard R, and Petrov V (1997) Cytosolic calcium changes induced by angiotensin II in human peripheral blood mononuclear cells are mediated via angiotensin II subtype 1 receptors. *J Hypertens* **15:**871–876.

Liu ECK, Hedberg A, Goldenberg HG, Harris DN, and Webb IM (1992) Dup 753, the selective angiotensin II receptor blocker, is a competitive antagonist to human platelet thromboxane A2/prostaglandin H2 (TP) receptors. *Prostaglandins* **44:**89–99.

Miettinen HM, Mills JS, Gripentrog JM, Dratz EA, Granger BL, and Jesaitis AJ (1997) The ligand binding site of the formyl peptide receptor maps in the transmembrane region. *J Immunol* **159:**4045–4054.

Murphy PM (1994) The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* **12:**593–633.

Nahmod VE, Dabsys SM, and Sterin-Princ AE (1992) Human monocytes produce angiotensin I and II and mediate the LPS stimulation of TNF-α release: effect of saralasin. *5th World Conference on Clinical Pharmacology and Therapeutics* A0. 27.04.

Noda K, Saad Y, Kinoshita A, Boyle TP, Graham RM, Husain A, and Karnik SS (1995) Tetrazole and carboxylate groups of angiotensin receptor antagonists bind to the same subsite by different mechanisms. *J Biol Chem* **270:**2284–2289.

Perez HD, Holmes R, Vilander LR, Adams RR, Manzana W, Jolley D, and Andrews WH (1993) Formyl peptide receptor chimeras define domains involved in ligand binding. *J Biol Chem* **268:**2292–2295.

Perez HD, Vilander L, Andrews WH, and Holmes R (1994) Human formyl peptide receptor ligand binding domain(s): studies using an improved mutagenesis/expression vector reveal a novel mechanism for the regulation of the receptor occupancy. *J Biol Chem* **269:**22485–22487.

Quehenberger O, Prossnitz ER, Cavanagh SL, Cochrane CG, and Ye RD (1993) Multiple domains of the N-formyl peptide receptor are required for high affinity ligand binding. *J Biol Chem* **268:**18167–18175.

Raiden S, Giordano M, Andonegui G, Trevani AS, López DH, Nahmod V, and Geffner JR (1997) Losartan, a selective inhibitor of subtype AT1 receptors for angiotensin II, inhibits the binding of N-formylmethionyl-leucyl-phenylalanine to neutrophil receptors. *J Pharmacol Exp Ther* **281:**624–628.

Raiden S, Pereyra Y, Nahmod V, Alvarez C, Castello L, Giordano M, and Geffner JR (2000) Losartan, a selective inhibitor of subtype AT1 receptors for angiotensin II, inhibits neutrophil recruitment in the lung triggered by fMLP. *J Leukoc Biol* **68:**700–706.

Regoli D, Park WK, and Rioux F (1974) Pharmacology of angiotensin. *Pharmacol Rev* **26:**69–123.

Reinhardt PH, Ward CA, Giles WR, and Kubes P (1997) Emigrated rat neutrophils adhere to cardiac myocytes via α_4 integrin. *Circ Res* **81:**196–201.

Shanley TP, Schmal H, Warner RL, Schmid E, Friedl HP, and Ward PA (1997) Requirements for C-X-C chemokines (macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant) in IgG immune complex-induced lung injury. *J Immunol* **158:**3439–3448.

Sinuff T, Cook DJ, Peterson JC, and Fuller HD (1999) Development, implementation and evaluation of a ketoconazole practice guideline for ARDS prophylaxis. *J Crit Care* **14:**1–6.

Slotman GJ, Burchard KW, D'Arezzo A, and Gann DS (1988) Ketoconazole prevents acute respiratory failure in critically ill surgical patients. *J Trauma* **28:**648–654.

Thies SD, Corbin RS, Goff CD, Binns OA, Buchanan SA, Shockey KS, Frierson HF, Young JS, and Tribble CG (1996) Thromboxane receptor blockade improves oxygenation in an experimental model of acute lung injury. *Ann Thorac Surg* **61:** 1453–1457.

Timmermans PB (1999) Angiotensin II receptor antagonists: an emerging new class of cardiovascular therapeutics. *Hypertens **Res*** **22:**147–153.

Timmermans PB, Wong PC, Chiu AT, Herblin WF, Benfield P, Carini DJ, Lee RJ, Wexler RR, Saye J, and Smith RD (1993) Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol Rev* **45:**205–251.

Ware LB and Matthay MA (2000) The acute respiratory distress syndrome. *N Engl J Med* **342:**1334–1349.

Weiland JE, Davis WB, Holter JF, Mohammed JR, Dorinsky PM, and Gadek JE (1986) Lung neutrophils in the adult respiratory syndrome. Clinical and pathophysiologic significance. *Am Rev Respir Dis* **133:**218–225.

Wisner D, Sturm J, Sutter G, Ellendorf B, and Nerlich M (1988) Thromboxane receptor blockade in an animal model of ARDS. *Surgery* **104:**91–97.

Wyncoll DL and Evans TW (1999) Acute respiratory distress syndrome. *Lancet* **354:**497–501.

Yu M and Tomasa G (1993) A double-blind, prospective, randomized trial of ketoconazole, a thromboxane synthetase inhibitor, in the prophylaxis of the adult respiratory distress syndrome. *Crit Care Med* **21:**1635–1642.

Zhang P, Summer WR, Bagby GJ, and Nelson S (2000) Innate immunity and pulmonary host defense. *Immunol Rev* **173:**39–51.

Address correspondence to: Jorge Geffner, Laboratorio de Inmunología, IIHEMA, Academia Nacional de Medicina, Pacheco de Melo 3081, 1425 Buenos Aires, Argentina. E-mail: geffnerj@fibertel.com.ar