EFFECT OF PD98059, A SELECTIVE MAPK3/MAPK1 INHIBITOR, ON ACUTE LUNG INJURY IN MICE

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The aim of the present study is to evaluate the contribution of mitogen-activated protein kinase 1-3 (MAPK3/MAPK1) in a model of acute lung inflammation in mice. Injection of carrageenan into the pleural cavity of mice elicited an acute inflammatory response characterized by: accumulation of fluid containing a large number of neutrophils (PMNs) in the pleural cavity, infiltration of PMNs in lung tissues and subsequent adhesion molecule expression (I-CAM and P-selectin), lipid peroxidation, and increased production of tumour necrosis factor-α, (TNF-α) and interleukin-1β (IL-1β). Furthermore, carrageenan induced lung apoptosis (Bax and Bcl-2 expression) as well as nitrotyrosine formation, NF-κB activation, and pJNK expression, as determined by immunohistochemical analysis of lung tissues and the degree of lung inflammation and tissue injury (histological score). Administration of PD98059, an inhibitor of MAPK3/MAPK1 (10 mg/kg) 1 h after carrageenan caused a reduction in all the parameters of inflammation measured. Thus, based on these findings we propose that inhibitors of the MAPK3/MAPK1 signaling pathways, such as PD98059, may be useful in the treatment of various inflammatory diseases.

The inflammatory process is invariably characterized by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and by the release of chemicals from tissues and migrating cells (1). Carrageenan-induced local inflammation is commonly used to evaluate anti-inflammatory effects of non-steroidal drugs (NSAIDs). Therefore, carrageenan-induced local inflammation (paw edema or pleurisy) is a useful model to assess the contribution of mediators involved in cellular alterations during the inflammatory process. The initial phase of acute inflammation (0-1 h), which is not inhibited by NSAIDs such as indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine (5-HT) and bradykinin, followed by a latent phase (1-6 h), mainly sustained by prostaglandin release and attributed to the induction of inducible cyclo-oxygenase (COX-2) in the tissue (2). It appears that the onset of carrageenan acute inflammation has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical, as well as to the release of other neutrophil-derived mediators (3). In this context, a crucial role is played by the endothelium, which modulates extravasation and permeability in response to inflammatory products,

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further favouring the development of inflammation. In particular, migration and accumulation of PMNs and macrophages is a complex phenomenon involving endothelium-based adhesion molecules such as Inter-Cellular Adhesion Molecule 1 (ICAM-1) and P-selectin (4). Several experimental findings have shown that these mediators contribute to tissue damage characteristic of the inflammatory process (5). In addition, the elevation of intracellular Ca^{2+} concentration causes release of proteases (e.g., leukocyte elastase or cathepsin G) and formation of reactive oxygen species (ROS) (6), both of which not only destroy invading particles but also damage cells and tissues of the host. Moreover, ROS generation induced by intracellular Ca^{2+} elevation causes lipid peroxidation and DNA single strand damage (7). Interference with the generation or action of these pro-inflammatory mediators exerts beneficial effects in a variety of inflammation models including the carrageenan-induced paw edema and pleurisy model (8). More recently, it was demonstrated that phosphorylation of ERK1/2 and p38 MAPK results in expression of genes mediating the inflammatory responses, such as tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) (9). Indeed, administration of p38 and c-Jun NH_2-terminal kinase (JNK) inhibitors partially rescued neurons from death in the lipopolysaccharide (LPS)-treated microglia-neuron co-culture (10), and inhibition of ERK1/2 activation may also reduce Interleukin 1 (IL-1)-induced cortical neuron damage (11). These findings suggest that initiation of inflammatory responses is related to activation of MAPKs, especially ERK1/2 and p38 MAPK. However, *in-vivo* evidence of strategies directed to blocking the initiation of this cascade linking MAPK activation in acute lung injury has not been fully delineated. In this study, the use of (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) (PD98059), a specific inhibitor of the activation of MAPK3/MAPK1 (10), allowed us to demonstrate that MAPK activation plays a key role in the modulation of acute lung injury. In particular, we determined the following endpoints of the inflammatory response: (i) polymorphonuclear (PMN) infiltration (assessing myeloperoxidase [MPO] activity), (ii) lipid peroxidation (malondialdehyde [MDA] levels), (iii) nitration of tyrosine residues as an indicator of peroxynitrite (by immunohistochemistry), (iv) inducible nitric oxide synthase (iNOS) expression, (v) nuclear factor *κB* (NF-κB) expression, (vi) apoptosis (Bax and Bcl-2 expression), and (vii) lung damage (histology).

MATERIALS AND METHODS

Animals

Male Adult CD1 mice (25-30g, Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Carrageenan-induced pleurisy

Carrageenan-induced pleurisy was induced as previously described (12). Mice were anaesthetized with isoflurane and subjected to a skin incision at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.1 ml) or saline containing 2% λ-carrageenan (0.1 ml) was injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were killed by inhalation of CO_2. The chest was carefully opened and the pleural cavity rinsed with 1 ml of saline solution containing heparin (5 U ml^{-1}) and indomethacin (10 μg ml^{-1}). The exudate and washing solution were removed by aspiration and the total volume measured. Any exudate, which was contaminated with blood, was discarded. The amount of exudate was calculated by subtracting the volume injected (1 ml) from the total volume recovered. The leukocytes in the exudate were suspended in phosphate-buffer saline (PBS) and counted with an optical microscope in a Burker's chamber after Blue Toluidine staining.

Experimental Design

Mice were randomized into 4 groups (n= 40 animals/group):

(i) CAR + vehicle group. Mice were subjected to carrageenan-induced pleurisy and received the vehicle for PD98059 (10% dimethylsulfoxide (DMSO) (v/v) i.p. bolus 1 h after carrageen administration (*N*=10);

(ii) *PD98059 group*. Same as the CAR + vehicle group but were administered PD98059 (10 mg kg^{-1} i.p. bolus) 1 h after carrageenan administration (*N*=10);

(iii) Sham+saline group. Sham-treated group in which identical surgical procedures to the CAR group were performed, except that the saline was administered instead of carrageenan (n=10);

(iv) Sham+ PD98059 *group.* Identical to Sham+saline group except for the administration of PD98059 (10 mg kg^{-1} i.p. bolus) 1 h after carrageenan administration of saline (*N*=10).

The doses of PD98059 (10 mg kg^{-1}) used here were based on previous *in vivo* studies that demonstrated a regulation of inflammation process (13-14).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in the lung tissues as previously described (15) at 4 h after carrageenan administration. At the specified time following pleurisy, lung tissues were obtained and weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 x g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37°C and was expressed as units of MPO/mg of proteins.

Immunohistochemical localisation of ICAM-1, P-Selectin, iNOS, nitrotyrosine, PAR, and phosphorylated c-Jun NH_2-terminal kinase JNK

At the end of the experiment, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with biotin and avidin (DBA, Milan, Italy), respectively. Sections were incubated overnight with 1) purified goat polyclonal antibody directed towards P-selectin which reacts with mice; or 2) with purified hamster anti-mouse ICAM-1 (CD54) (1:500 in PBS, w/v) (DBA, Milan, Italy) or 3) with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, v/v) or with anti-PAR antibody (1:500 in PBS, v/v) or 4) with anti-iNOS antibody (1:500 in PBS, v/v), or 5) anti- phosphorylated JNK antibody (1:500 in PBS, v/v). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). In order to confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. To verify the binding specificity for ICAM-1, P-selectin, iNOS, and pJNK, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections, indicating that the immunoreaction was positive in all the experiments carried out.

Histological examination

Lung tissue samples were taken 4 h after injection of carrageenan. Lung tissue samples were fixed for 1 week in 10% (w/v) PBS-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were then de-paraffinized with xylene, stained with hematoxylin and eosin. All sections were studied using Axiovision Ziess (Milan, Italy) microscope. The following morphological criteria were used for scoring: 0, normal lung; grade 1, minimal edema or infiltration of alveolar or bronchiolar walls; grade 3, moderate edema and inflammatory cell infiltration without obvious damage to lung architecture; grade 4, severe inflammatory cell infiltration with obvious damage to lung architecture. All the histological studies were performed in a blinded manner.

Measurement of cytokines

TNF-α and IL-1β levels were evaluated in the exudate 4 h after the induction of pleurisy by carrageenan injection as previously described (16). The assay was carried out using a colorimetric commercial ELISA kit (Calbiochem-Novabiochem Corporation, Milan, Italy) with a lower detection limit of 10 pg/ml.

Measurement of nitrite-nitrate concentration

Total nitrite in exudates, an indicator of nitric oxide (NO) synthesis, was measured as previously described (17). Briefly, the nitrate in the sample was first reduced to nitrite by incubation with nitrate reductase (670 mU ml^{-1}) and β-nicotinamide adenine dinucleotide 3'-phosphate (NADPH) (160 μM) at room temperature for 3 h. The total nitrite concentration in the samples was then measured using the Griess reaction, by adding 100 μl of Griess reagent (0.1% (w/v) naphthylethylendiamide dihydrochloride in H_2O and 1% (w/v) sulphanilamide in 5% (v/v) concentrated H_3PO_4; vol. 1:1) to the 100 μl sample. The optical density at 550 nm (OD_{550}) was measured using ELISA microplate reader (SLT-Lab Instruments, Salzburg, Austria). Nitrite concentrations

were calculated by comparison with OD_{550} of standard solutions of sodium nitrite prepared in H_2O.

Western blot analysis for IκB-α, NF-κB p65, Bax, Bcl-2, pERK-1/2, ERK-2, and p38

Cytosolic and nuclear extracts were prepared as previously described (18) with slight modifications. Briefly, lung tissues from each mouse were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1,000 x g for 10 min at 4°C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM TRIS-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 μm leupeptin, 0.2 mM sodium orthovanadate. After centrifugation for 30 min at 15,000 x g at 4°C, the supernatants containing the nuclear protein were stored at -80 for further analysis. The levels of IκB-α, phospho-NF-κB p65 (serine 536), phospho-SAPK/JNK, phospho-p38 MAP Kinase, Bax, and Bcl-2 were quantified in cytosolic fraction from lung tissue collected 4 h after carrageenan injection, while NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1x PBS, 5% (w/v) non-fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs IκB-α (Santa Cruz Biotechnology, 1:1000), or phospho-NF-κB p65 (serine 536) (Cell Signaling,1:1000), or anti-Bax (1:500; Santa Cruz Biotechnology), or anti-Bcl-2 (1: 500; Santa Cruz Biotechnology), or anti-ERK-2, (1:1000 Santa Cruz Biotechnology) or anti-pERK1/2 (1:1000 Santa Cruz Biotechnology) or anti-NF-kB p65 (1:1000; Santa Cruz Biotechnology) or anti-phospho-p38 MAP Kinase (Thr180/Tyr182) (1:1000; Cell Signaling) in 1x PBS, 5% w/v non-fat dried milk, 0.1% Tween-20 (PMT) at 4°C, overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature.

To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against α-tubulin protein (1:10,000 Sigma-Aldrich Corp.). The relative expression of the protein bands of IκB-α (~37 kDa), NF-kB p65 (75kDa), Bax (~23 kDa), Bcl-2 (~29 kDa), phospho-p38 MAP Kinase (43 kDa) was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM), and standardized for densitometric analysis to β-actin levels.

The dual-phosphorylated form of ERK (p-ERK) antibody identified two bands of approximately 44 and 42 kDa (corresponding to p-ERK1 and p-ERK2, respectively). The anti-ERK2 antibody detects total ERK2 (i.e. detects both phosphorylated and non-phosphorylated forms of ERK2).

Thiobarbituric acid-reactant substance measurement

Thiobarbituric acid-reactant substance measurement, which is considered a highly useful indicator of lipid peroxidation, was determined in the lung tissue 4 h after carrageenan as previously described (19). Thiobarbituric acid-reactant substances were calculated by comparison with OD_{650} of standard solutions of 1,1,3,3-tetramethoxypropan 99% malondialdehyde bis (dymethyl acetal) 99% (MDA) (Sigma, Milan). The absorbance of the supernatant was measured by spectrophotometer at 650 nm. MDA quantities were calculated by linear regression analysis of the standard curve. Values were expressed as μM MDA/mg of proteins.

Materials

All compounds were obtained from Sigma-Aldrich Company Ltd. (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) of *n* observations. For the *in vivo* studies *n* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissue sections collected from all the animals in each group. The results were analyzed by one-way ANOVA followed by a Bonferroni *post-hoc* test for multiple comparisons. A *p*-value less than 0.05 was considered significant. The mean values from individual group means were then compared with Student's unpaired t test. A *P*-value of less than 0.05 was considered significant.

RESULTS

Effects of PD98059 on carrageenan-induced pleurisy

When compared to lung sections taken from saline-treated animals (sham group Fig. 1 panel A,A), histological examination of lung sections taken from mice treated with carrageenan revealed significant tissue damage and edema (Fig. 1 panel

Table I. *Effect of PD98059 on carrageenan-induced inflammation, TNF-α and IL-1β production in the pleural exudate.*

	Volume Exudate (ml)	PMNs infiltration (million cells/mouse)	TNF-α (pg/ml)	IL-1β (pg/ml)
Sham + Vehicle	0.045 ± 0.03	0.4 ± 0.14	3.0 ± 0.5	5.0 ± 1.1
Sham + PD98059 (10 mg kg-1)	0.057 ± 0.02	0.58± 0.2	4.0 ± 0.6	6.0 ± 1.1
CAR + Vehicle	1.21 ± 0.12*	7.4± 0.35*	48 ± 6.5*	78 ± 5.9*
CAR + PD98059 (10 mg kg-1)	0.25 ± 0.1°	1.4 ± 0.38°	12.5 ± 4.5°	21 ± 3.5°

*TNF and IL-1 exudate levels are based on the actual exudate volume generated in the different experimental arms. Data are means±s.e. means of 10 mice for each group. *P<0.01 versus sham. °P<0.01 versus carrageenan.*

A,B), as well as infiltration of neutrophils (PMNs) within the tissues. PD98059 (10 mg kg^{-1}) reduced the degree of lung injury (Fig. 1 panel A,C). Furthermore, injection of carrageenan elicited an acute inflammatory response characterized by the accumulation of fluid (edema) in the pleural cavity (Table I) containing large amounts of PMNs (Table I). Treatment with PD98059 attenuated carrageenan-induced edema formation and PMN infiltration (Table I).

Effects of PD98059 on the expression of adhesion molecules (ICAM-1, P-selectin)

Four hours after carrageenan injection, the staining intensity for ICAM-1 substantially increased along the vessels (Fig. 1 panel B,A see densitometry analysis 1E) mainly localized in the vascular endothelium (Fig. 1 panel B,A). Lung tissue sections obtained from carrageenan-treated mice showed positive staining for P-selectin localized in the vessels (Fig. 1 panel B,C see densitometry analysis 1F). No positive staining for ICAM-1 or P-selectin was observed in the lungs of carrageenan-treated mice pre-treated with PD98059 (10 mg kg^{-1}) (Fig. 1 panel B,B,D respectively see densitometry analysis (Fig. 1 panel B,E and F).

Effects of PD98059 on carrageenan-induced NO production

Immunohistochemical analysis of lung sections obtained from carrageenan-treated mice revealed positive staining for iNOS (Fig. 2 panel A, A see densitometry analysis panel A, C). In contrast, no staining for iNOS was found in the lungs of carrageenan-treated mice that had been treated with PD98059 (10 mg kg^{-1}) (Fig. 2 panel A, B see densitometry analysis panel A, C). A significant increase in iNOS expression 4 h after carrageenan injection, as assayed by Western blot analysis, was also detected in lungs obtained from mice subjected

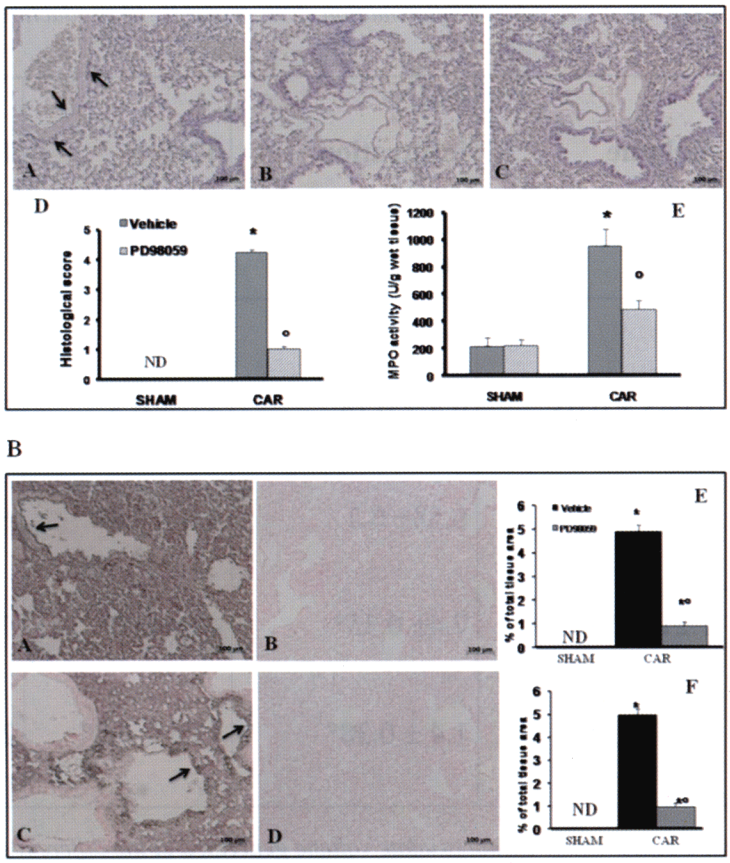
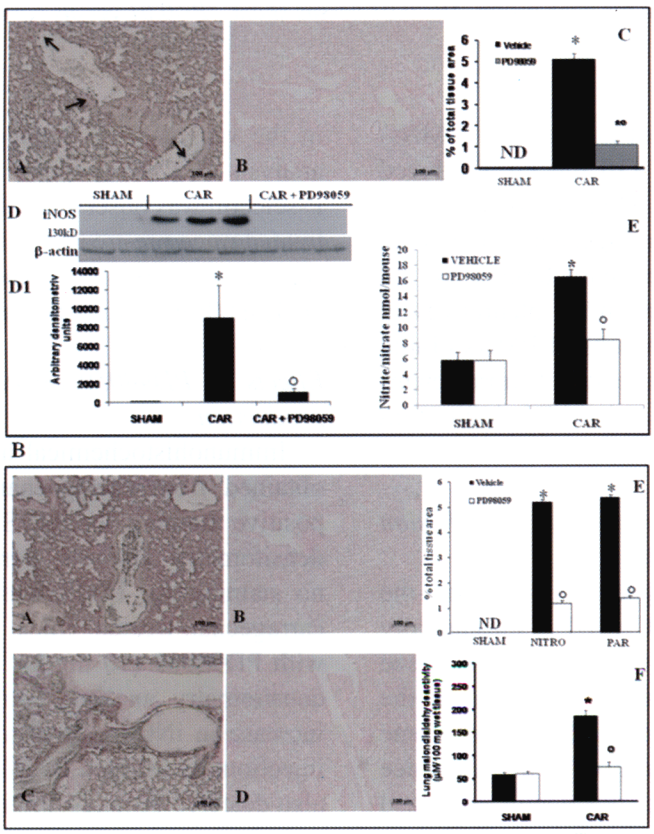

A
B
C
D
Vehicle
PD98059
Histological score
ND
SHAM
CAR
E
MPO activity (U/g wet tissue)
SHAM
CAR
B
A
B
C
D
E
% of total tissue area
ND
SHAM
CAR
F
% of total tissue area
ND
SHAM
CAR

Fig.1


A
B
C
SHAM
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D
iNOS
130kD
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Arbitrary densitometric units
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E
VEHICLE
PD98059
Nitrite nitrate nmol/mouse
SHAM
CAR
B
A
B
C
D
E
Vehicle
PD98059
% total Isnitr area
ND
SHAM
NITRO
PAR
F
Lung malondialdehyde activity (µM/100 mg wet tissue)
SHAM
CAR

Fig.2

Fig. 1. *Effect of PD98059 on lung injury and immunohistochemical localization of ICAM-1 and P-selectin in the lung. When compared with lung sections taken from sham-treated animals (panel **A**, A), lung sections from carrageenan-treated mice (panel **A**, B) demonstrated edema and PMN infiltration (see arrows). Lung sections from carrageenan-treated mice which received PD98059 exhibited reduced tissue injury and inflammatory cell infiltration (panel **A**, C). MPO activity, an index of neutrophil infiltration, was significantly increased in lung tissues 4 h after carrageenan (CAR) administration (panel **A**, **F**). PD98059 significantly reduced the carrageenan-induced elevation of MPO tissues levels. The figure is representative of at least three experiments performed on different experimental days. Data are expressed as mean±SEM from n*=10 mice for each group. $^{*}P<0.01$ versus sham group. $^{\circ}P<0.01$ versus carrageenan. Immunohistochemical analyses of sections from carrageenan-treated mice showed positive staining (see arrows) for ICAM-1 (panel **B**, A) and P-selectin (panel **B**, C). The intensity of this staining was markedly reduced in tissue sections obtained from PD98059-treated mice (panel **B**, B, D). The figures are representatives of all the animals in each group. Densitometry analysis of lung immunocytochemistry photographs (n=5) for ICAM-1 and P-selectin (panel **B**, E, F). The assay was carried out by using Optilab Graftek software. Data are expressed as percentage of total tissue area.* $^{*}P<0.01$ versus sham group. $^{\circ}P<0.01$ versus carrageenan.

Fig. 2. *Effect of PD98059 on carrageenan-induced iNOS expression and nitrotyrosine and PAR formation in the lung. Lung sections taken from carrageenan-treated mice treated with vehicle showed positive staining (see arrows) for iNOS, localized mainly in inflammatory cells (panel **A**, **A**). The degree of positive staining for iNOS was markedly reduced in tissue sections obtained from mice treated with PD98059 (panel **A**, **B**).Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for iNOS (panel **A**, C) was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. A significant increase in iNOS (panel **A**, **D,D1**) expression, assayed by Western blot analysis, was detected in lungs obtained from mice subjected to carrageenan-induced pleurisy. Treatment with PD98059 significantly attenuated iNOS (panel **A**, **D,D1**) expression in the lung tissues. Nitrite and nitrate levels, stable NO metabolites, were significantly increased in the pleural exudates at 4 h after carrageenan (CAR) administration (panel **A**, E). PD98059 significantly reduced the carrageenan-induced elevation of nitrite and nitrate exudates levels. (panel **A**, E) The figure is representative of at least three experiments performed on different experimental days. Data are expressed as mean±SEM from n*=10 mice for each group. $^{*}P<0.01$ versus sham group. $^{\circ}P<0.01$ versus carrageenan. Lung sections taken from carrageenan-treated mice treated with vehicle showed positive staining for nitrotyrosine and PAR, localized mainly in inflammatory cells (panel **B**, A, C). There was a marked reduction in the immunostaining for nitrotyrosine and PAR in the lungs of carrageenan-treated PD98059 (panel **B**, **B**, **D**). Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for nitrotyrosine and PAR (panel **B**, E) was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area.MDA levels, an index of lipid peroxidation, were significantly increased in lung tissues 4 h after carrageenan (CAR) administration (panel **B**, **F**). PD98059 significantly reduced the carrageenan-induced elevation of MDA tissues levels. The figure is representative of at least three experiments performed on different experimental days. Data are expressed as mean±SEM from n*=10 mice for each group. $^{*}P<0.01$ versus sham group. $^{\circ}P<0.01$ versus carrageenan.

to carrageenan-induced pleurisy (Fig. 2 panel A, D see densitometry analysis panel A, D1). PD98059 (10 mg kg^{-1}) treatment significantly attenuated this iNOS expression (Fig. 2 panel A, D see densitometry analysis panel A, D1). NO levels were also significantly increased in the exudate obtained from mice administered carrageenan (Fig. 2 panel A, E). Treatment of mice with PD98059 significantly reduced NO exudates levels (Fig. 2 panel A, E).

Effects of PD98059 on carrageenan-induced nitrotyrosine formation and PAR production

Immunohistochemical analysis of lung sections obtained from the mice treated with carrageenan revealed positive staining for nitrotyrosine (Fig. 2 panel B, A see densitometry analysis panel B, E). In contrast, no positive staining for nitrotyrosine was found in the lungs of carrageenan-treated mice, which had been treated with PD98059 (10 mg kg^{-1})

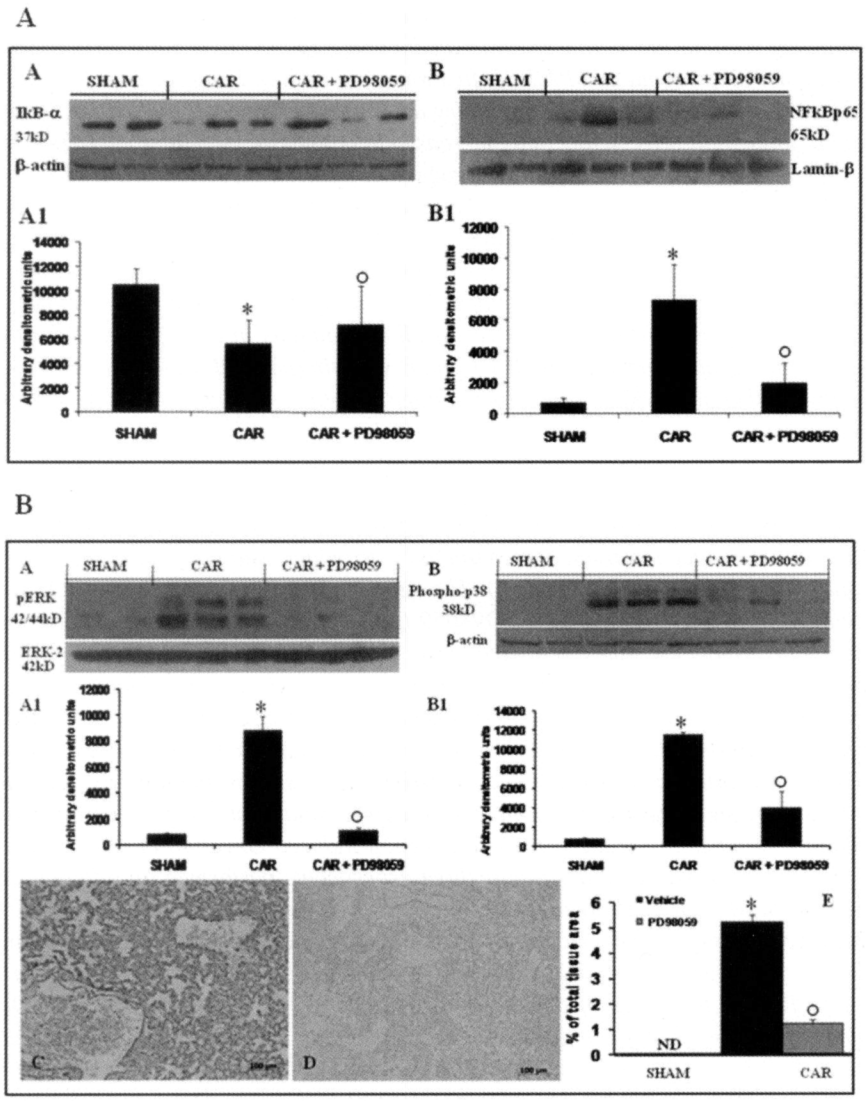

Fig. 3. *Effects of PD98059 treatment on NF-κB and activated kinases. Representative Western blots showing the effects of PD98059 on IκB-α* degradation (panel ***A, A,A1)*** *and phosphorylation of Ser536 on NF-κB* subunit p65 (panel ***A**, B,B1)* *after carrageenan (CAR) injection. A representative blot of lysates (panel **A, A,B**) obtained from from mice is shown and densitometry analysis of all animals is reported. The results in panel **A, A1, B1** are expressed as mean±SEM from n*=10 mice for each group $^{*}P<0.01$ versus carrageenan. Representative Western blots showing the effects of PD98059 on the dual-phosphorylated form of ERK or active ERK1/2 (pERK1/2) and total ERK1/2 (ERK2). pERK1/2 is up regulated in injured mice as compared to sham-operated mice (panel ***B**, A and A1). pERK1/2 level were significantly attenuated in PD98059 (10 mg kg-1)- treated mice in comparison to carrageenan-treated mice (panel **B**, A, A1). Moreover, pleurisy caused also a significant increase in phospho-p38 expression at 4 h after injection (panel **B**, B,B1). The treatment with PD98059 significantly reduced p38 expression (panel **B**, B,B1). The relative expression of the protein bands was standardized for densitometric analysis to b-actin levels, and reported in (panel **B**, A1,B1. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days. *p<0.01 vs. Sham. °p<0.01 vs carrageenan +vehicle. Representative sections showing the expression and distributions of p-JNK by using phospho-antibodies, as shown in carrageenan-treated mice (panel **B**, C) and PD98059 (10 mg kg-1)- treated mice (panel **B**, D). Immunohistochemical staining for p-JNK showing diffuse staining mainly localized in inflammatory cells Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for p-JNK (panel **B**, E) was assessed.*

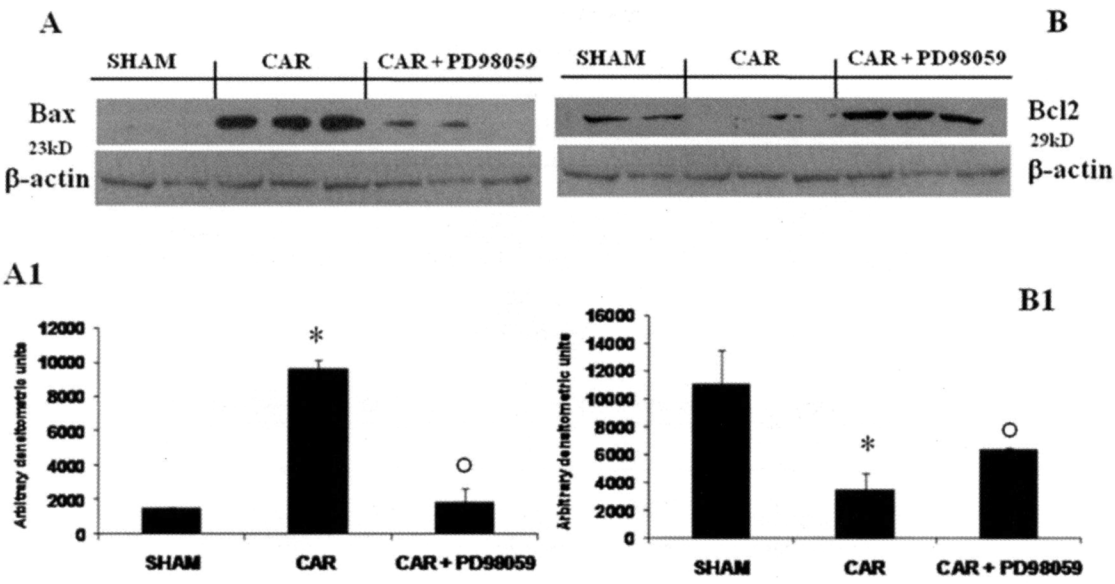

Fig. 4. *Effect of PD98059 on Bax and Bcl-2 expression. By Western blot analysis, Bax levels were appreciably increased in the carrageenan-treated mice (panels **A** and A1). On the contrary, PD98059 treatment prevented the carrageenan-induced Bax expression (panels **A** and A1). Moreover, a basal level of Bcl-2 expression was detected in lung from sham-operated mice (panels **B** and B1). At 4 h after carrageenan injection, Bcl-2 expression was significantly reduced in lung from carrageenan-treated mice (panels **B** and B1). PD98059 treatments significantly reduced the carrageenan-induced inhibition of Bcl-2 expression (panels **B** and B1).The relative expression of the protein bands was standardized for densitometric analysis to b-actin levels, and reported in panel A1 and B1 are expressed as mean±SEM from n=5/6 lungs for each group. *P<0.01 versus sham, °P<0.01 versus carrageenan +vehicle.*

(Fig. 2 panel B, B see densitometry analysis panel B, E). Immunohistochemistry for PAR, as an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR in inflammatory cells in the lung tissues of carrageenan-treated mice (Fig. 2 panel B, C see densitometry analysis panel B, E). PD98059 treatment (10 mg kg^{-1}) reduced the degree of positive staining for PAR (Fig. 2 panel B, D see densitometry analysis panel B, E) in the lung tissues.

In addition, at 4 h after carrageenan-induced pleurisy, MDA levels were also measured in the lungs as an indicator of lipid peroxidation. As shown in Fig 2 panel B, F, MDA levels were significantly increased in the lungs of carrageenan-treated mice. Lipid peroxidation was significantly attenuated by the intraperitoneal injection of PD98059 (Fig. 2 panel B, F).

Effects of PD98059 on the release of pro-inflammatory cytokine induced by carrageenan

When compared to sham animals, the injection of carrageenan resulted in an increase in the levels of TNF-α and IL-1β in the pleural exudates (Table I). The release of TNF-α and IL-1β was attenuated by treatment with PD98059 (Table I).

Effect of PD98059 on IκB-α degradation and NF-κB p65 activation

We evaluated IκB-α degradation and nuclear NF-κB p65 by Western blot analysis to investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of carrageenan-induced pleurisy

A basal level of IκB-α was detected in the lung tissues of sham-animals (Fig. 3 panel A, A see densitometry analysis panel A, A1), whereas in carrageenan-treated mice IκB-α levels were substantially reduced (Fig. 3 panel A, A see densitometry analysis panel A, A1). PD98059 prevented carrageenan-induced IκB-α degradation,

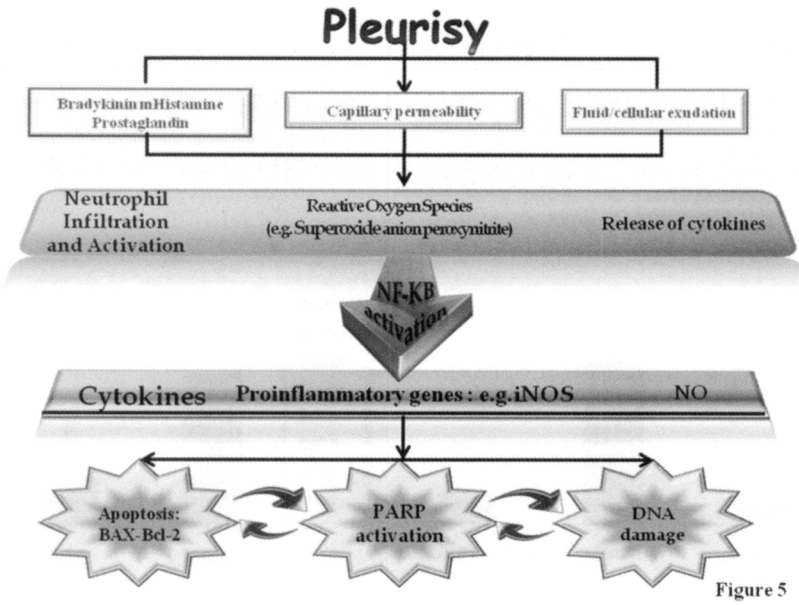

Fig. 5. *Pleurisy pathophysiology.*

the IκB-α levels observed in these animals were similar to those of the sham group (Fig. 3 panel A, A see densitometry analysis panel A, A1). In addition, carrageenan administration caused a significant increase in the NF-kB p65 levels in the nuclear fractions from lung tissues (Fig. 3 panel A, B see densitometry analysis panel A, B1)compared to the sham-treated mice (Fig. 3 panel A, B see densitometry analysis panel A, B1). PD98059 treatment significantly reduced the levels of NF-kB p65 as shown in (Fig. 3 panel A, B see densitometry analysis panel A, B1).

Effect of PD98059 on the activation of MAPK pathways and the expression of phospho-p38 after carrageenan-induced pleurisy

To investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of carrageenan-induced pleurisy, we also evaluated the phosphorylation of ERK1/2 which results in expression of pro-inflammatory genes mediating the inflammatory responses characteristic of carrageenan-induced pleurisy. The activation of MAPK pathways, in particular the phosphorylation of ERK1/2 expression, was investigated by Western blot in lung tissues homogenates at 4 h after carrageenan administration. A significant increase in pERK1/2 levels was observed in carrageenan-treated mice (Fig. 3 panel B, A see densitometry analysis panel B, A1). The treatment of mice with PD98059 significantly reduced the level of pERK1/2 (Fig. 3 panel B, A see densitometry analysis panel B, A1). In addition, a marked positive immunostaining for the phosphorylated JNK was found mainly localized in inflammatory cells in the lung tissues collected from vehicle-treated mice at 4 h after carrageenan administration (Fig. 3 panel B, C see densitometry analysis panel B, E). PD98059 treatment reduced the degree of the expression of JNK (Fig. 3 panel B, D see densitometry analysis panel B, E) (Fig. 6D, see densitometry analysis Fig. 6E). Sections of lung tissues obtained from the sham mice did not reveal any immunoreactivity for phosphorylated JNK (data not shown). Moreover, we evaluated the phospho-p38 expression by Western blot analysis to further investigate the cellular mechanisms by which treatment with PD98059 may attenuate the development of acute lung inflammation. At 4 h after carrageenan administration, a significant increase in the phospho-p38 expression was observed in vehicle-treated mice (Fig. 3 panel B, B see densitometry analysis panel B, B1). The treatment with PD98059 significantly reduced the p38 expression (Fig. 3 panel B, B see densitometry analysis panel B, B1).

Effects of PD98059 on Bax and Bcl-2 expression

The presence of Bax in lung homogenates was investigated by Western blot 4 h after carrageenan administration. A basal level of Bax was detected in lung tissues obtained from sham-treated animals (Fig. 4A, see densitometry analysis Fig 4A1). Bax levels were substantially increased in the lung tissues from carrageenan-treated mice (Fig. 4A, see densitometry analysis Fig. 4A1). On the contrary, PD98059 (10 mg kg^{-1}) treatment prevented the carrageenan-induced Bax expression (Fig. 4A, see densitometry analysis Fig. 4A1).

To detect Bcl-2 expression, whole extracts from lung tissues of mice were also analyzed by Western blot analysis. A basal level of Bcl-2 expression was detected in lung tissues from sham-treated mice (Fig. 4B, see densitometry analysis Fig. 4B1). At 4 hours after carrageenan administration, Bcl-2 expression was significantly reduced (Fig. 4B, see densitometry analysis Fig. 4B1). Treatment of mice with PD98059 (10 mg kg^{-1}) significantly attenuated carrageenan-induced inhibition of Bcl-2 expression (Fig. 4B, see densitometry analysis Fig. 4B1).

DISCUSSION

MAPKs are a family of serine-threonine kinases that mediate the nuclear response of cells to a wide variety of extracellular stresses such as inflammatory cytokines, growth factors, UV light, and osmotic stress (20). MAPKs are divided into 3 major subfamilies: the ERKs, of which ERKs 1 and 2 are the most abundant in mammalian cells, the JNKs, and the p38 MAPKs. Each MAPK is activated through dual phosphorylation via a specific upstream phosphorylation cascade. MAPKs have been implicated in the pathogenesis of asthma-like Th2 inflammation with chemical inhibition of ERK1/2 MAPK–reducing lung eosinophilia in ovalbumin-sensitized and -challenged rats and mice (21).In this report we investigated the effects of PD98059, an inhibitor of MAPK3/MAPK1, in a mouse model of carageenan-elicited acute lung inflammation. We were able to demonstrate that PD98059 attenuated: (i) the development of carrageenan-induced pleurisy, (ii) the infiltration of PMN's into the lung, (iii) the degree of lipid peroxidation in the lung, (iv) the expression of ICAM-1 and P-selectin, (v) pro-inflammatory cytokines production, (vi) the nitration of tyrosine residues, (vii) iNOS expression (vii) NF-κB expression (ix) apoptosis (x) Bax and Bcl-2 expression and (xi) the degree of lung injury caused by injection of carrageenan. All of these findings support the view that PD98059 markedly prevented the degree of acute inflammation in this mouse model.

Mechanistically, the ERK1/2 and p38 MAPK signaling pathways have been found to be involved in IL-13–induced lung inflammation and remodeling *in-vivo* (22). Previous studies show that the expression of activated ERK1/2 and p38 MAPK may play a key role in production of inflammatory cytokines and free radicals, such as NO (22). Recent evidence suggests that the activation of NF-κB may also be under the control of oxidant/antioxidant balance. Moreover, various experimental evidence has clearly suggested that NF-κB plays a central role in the regulation of many genes responsible for the generation of mediators or proteins in acute lung inflammation associated with carrageenan administration (23). NF-κB is normally sequestered in the cytoplasm, bound to regulatory protein IκBs. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IκB is phosphorylated by the enzyme IκB kinase (24). The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. The exact mechanisms by which PD98059 suppress NF-κB activation in inflammation are not known. We report here that carrageenan administration caused a significant increase in the nuclear translocation of the subunit p65 in the lung tissues at 4 h after carrageenan administration, whereas PD98059 treatment significantly reduced the NF-κB translocation. Moreover, we also demonstrate that the PD98059 treatment inhibited the IκB-α degradation. Taken together, the balance between pro-inflammatory and pro-survival roles of NF-κB may depend on the phosphorylation status of p65, and MAPK play a central role in this process. These observations are in agreement with a previous *in-vivo* study in which we clearly showed that the treatment with PD98059, prevented spinal cord injury elevation in p65 translocation and IκB-α degradation (14). NF-κB plays a central role in the regulation of many

genes responsible for the generation of mediators or proteins in inflammation including TNF-α, IL-1β and iNOS (25). There is good evidence that TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process (26). Various studies have clearly reported that inhibition of TNF-α formation significantly prevents the development of the inflammatory process (27). We have clearly confirmed a significant increase in the TNF-α and IL-1β production in the pleural exudates of carrageenan-treated mice. On the contrary, a significant reduction of TNF-α and IL-1β production was observed in the pleural exudates obtained from carrageenan-treated mice which received PD98059, suggesting that MAPK3/MAPK1 pathway plays an important role in the regulation of proinflammatory cytokines. This observation is in agreement with a previous study in which the inhibition of ERK 1/2 pathway activation may also reduce production of pro-inflammatory cytokine (14). As expected, a significant decrease of pERK1/2 levels was observed in the lung tissue obtained from carrageenan-treated mice which received PD98059 (23).

Furthermore, we observed that acute lung inflammation (4 h after carrageenan administration) induced the appearance of P-selectin on the endothelial vascular wall and up-regulated the surface expression of ICAM-1 on endothelial cells. Treatment with PD98059 abolished the expression of P-selectin and the up-regulation of ICAM-1 without effecting constitutive levels of ICAM-1 on endothelial cells. These results demonstrate that inhibition of the ERK 1/2 pathway may interrupt the interaction neutrophils and endothelial cells both at the early rolling phase mediated by P-selectin and at the late firm adhesion phase mediated by ICAM. The absence of an increased expression of the adhesion molecule in the lung tissue of carrageenan-treated mice administered PD98059 correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme MPO and with the attenuation of the lung tissue damage as evaluated by histological examination. Activation and accumulation of leukocytes is one of the initial events of tissue injury due to the release of oxygen free radicals, arachidonic acid metabolites and lysosomal proteases (28).

Enhanced formation of NO by iNOS may contribute to the inflammatory process (3). This study demonstrates that PD98059 attenuates the expression of iNOS in the lung in carrageenan-treated mice. This reduction in the expression of iNOS by PD98059 may contribute to the attenuation of nitrotyrosine formation and lipid peroxidation in the lung in carrageenan-treated animals. Nitrotyrosine formation, along with its detection by immunostaining, was initially proposed as a relatively specific marker for the detection of the endogenous formation "footprint" of peroxynitrite (29). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration e.g. reaction of nitrite with hypoclorous acid and the reaction of MPO with hydrogen peroxide can lead to the formation of nitrotyrosine (30). Increased nitrotyrosine staining is therefore considered as an indicator of "increased nitrosative stress" rather than a specific marker of the generation of peroxynitrite. ROS and peroxynitrite produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS resulting in the depletion of its substrate NAD *in vitro* and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. This process has been termed 'the PARS Suicide Hypothesis'. There is recent evidence that the activation of PARS may also play an important role in stress-related lung diseases such as asbestosis, silicosis, and acute respiratory distress syndrome. (31) Similarly, the protective effects of PARP inhibition and the PARP-1 knock out phenotype in asthma models have been attributed to inhibition of oxidative stress-induced cell dysfunction and tissue injury. We demonstrate here that PD98059 attenuates the increase in PARP activity caused by carrageenan-induced pleurisy.

Generation of free radicals and nitric oxide by activated macrophages has also been implicated in causing oligodendrocyte apoptosis (32).

We identified pro-apoptotic transcriptional changes, including up-regulation of pro-apoptotic Bax and down-regulation of anti-apoptotic Bcl-2, using Western blot assay. In the present study we report for the first time that the treatment with PD98059 in acute lung injury documents features of apoptotic cell death after carrageenan administration,

suggesting that protection from apoptosis may be a prerequisite for anti-inflammatory approaches. In particular, we demonstrated that the treatment with PD98059 lowers the signal for Bax in the treated group when compared with lung sections obtained from carrageenan-treated mice while, on the contrary, the signal is much more expressed for Bcl-2 in PD98059 treated mice than in carrageenan-treated mice. However, it is not possible to exclude that the anti-apoptotic effect observed after PD98059 treatment may be partially dependent on the attenuation of the inflammatory-induced damage. Further studies are needed in order to clarify these mechanisms.

Finally, in this study we demonstrate that PD98059 treatment significantly reduced acute lung inflammation using a plethora of end-point markers. The results of the present study enhance our understanding of the role of MAPK3/MAPK1 pathway in the pathophysiology of acute lung tissue injury following inflammation, implying that inhibitors of this pathway might represent a highly feasible pharmacotherapy in preventing inflammation and trauma.

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