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Effects of GW274150, a novel and selective inhibitor of iNOS activity, in acute lung inflammation

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1 The aim of this study was to investigate the effect of GW274150, a novel, potent and selective inhibitor of inducible nitric oxide synthase (iNOS) activity in a model of lung injury induced by carrageenan administration in the rats.

2 Injection of carrageenan into the pleural cavity of rats elicited an acute inflammatory response characterized by: fluid accumulation in the pleural cavity which contained a large number of polymorphonuclear cells (PMNs) as well as an infiltration of PMNs in lung tissues and subsequent lipid peroxidation, and increased production of nitrite/nitrate (NO_x), tumour necrosis factor α (TNF-α) and interleukin-1β (IL-1β).

3 All parameters of inflammation were attenuated in a dose-dependent manner by GW274150 (2.5, 5 and 10 mg kg^{-1} injected i.p. 5 min before carrageenan).

4 Carrageenan induced an upregulation of the intracellular adhesion molecules-1 (ICAM-1), as well as nitrotyrosine and poly (ADP-ribose) (PAR) as determined by immunohistochemical analysis of lung tissues.

5 The degree of staining for the ICAM-1, nitrotyrosine and PAR was reduced by GW274150. These results clearly confirm that NO from iNOS plays a role in the development of the inflammatory response by altering key components of the inflammatory cascade.

6 GW274150 may offer a novel therapeutic approach for the management of various inflammatory diseases where NO and related radicals have been postulated to play a role.
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Keywords: Pleurisy; adhesion molecules; peroxynitrite; inflammation; iNOS; reactive oxygen species; rat

Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony-stimulating factor; ICAM, intercellular adhesion molecules; IL, interleukin; INF, interferon; iNOS, inducible nitric oxide synthase; MDA, malondialdehyde; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide 3′-phosphate; NF, nuclear factor; NO, nitric oxide; NSAID, nonsteroidal anti-inflammatory drugs; PARS, poly (ADP-ribose) synthase; PBS, phosphate-buffered saline; PMNs, polymorphonuclear cells; PMSF, phenylmethylsulphonyl fluoride; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; STAT, signal transducer and activator of transcription; TNF, tumour necrosis factor

Introduction

Nitric oxide (NO) is a pleiotropic mediator, which acts in a variety of physiological and pathophysiological processes (Dinerman, 1993; Nathan, 1994; Szabò, 1995; Southan & Szabò, 1996). NO is produced from the oxidation of L-arginine by the enzyme NO synthase (Moncada & Palmer, 1991; Moncada and Higgs, 2002) which occurs in three major isoforms; two are constitutive (endothelial and neuronal, indicated with cNOS), and one is inducible (macrophagic). The constitutively expressed enzyme (cNOS) are calcium-dependent, release NO under physiological condition in various cells, including endothelial cells and neurons, and NO released by these isoforms are involved in the regulation of blood pressure in organ blood flow distribution, in the inhibition of the adhesion and activation of platelets and polymorphonuclear granulocytes and in neuronal transmission. The inducible isoform of NOS (iNOS) is calcium-independent and can be induced by proinflammatory agents, such as endotoxins (bacterial lipopolysaccharide, LPS), interleukin-1β, tumour necrosis factor-α (TNF-α) and interferon-γ (INF-γ), in endothelial and smooth-muscle cells, in macrophages and in other cell types (Moncada & Palmer, 1991; Dinerman, 1993; Nathan, 1994; Szabò, 1995; Southan & Szabò, 1996). Enhanced formation of NO following the induction of iNOS has been implicated in the pathogenesis of shock and inflammation (Nathan, 1994).

Recently it has been demonstrated that iNOS inhibitor prevents the activation of poly (ADP ribose) synthetase

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(PARS), and prevents the organ injury associated with inflammation. Although the severity and duration of inflammation may dictate the timing and extent of NOS expression, it is now evident that the upregulation of NOS can take place during sustained inflammation. Pharmacological inhibition of iNOS or genetic inactivation of NOS (iNOS knockout mice) attenuates the activation of the transcription factors nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription-3 (STAT-3), and ameliorates the increases in granulocyte colony-stimulating factor (G-CSF) messenger RNA levels in the tissue. Thus, induced nitric oxide, in addition to being a 'final common mediator' of inflammation, is essential for the upregulation of the inflammatory response. Furthermore, a picture of a pathway is evolving that contributes to tissue damage both directly *via* the formation of peroxynitrite, with its associated toxicities, and indirectly through the amplification of the inflammatory response. Some authors proposed the involvement of peroxynitrite in mediating cell damage during various pathophysiological condition like inflammation or oxidant stress (Salvemini *et al*., 1996a; Cuzzocrea *et al*., 2001) and it has been recently suggested that some of the cytotoxic effects of NO are tightly related to the production of peroxynitrite, an high-energy oxidant deriving by the rapid reaction of NO with superoxide (Beckman *et al*., 1990; Crow & Beckman, 1995; Pryor & Squadrito, 1995). The resulting oxidative stress may cause cell death and tissue damage that characterize a number of human disease states like neurological disorders and stroke, inflammatory bowel disease, arthritis, toxic shock and acute reperfusion injuries (Yamada & Grisham, 1991; Cuzzocrea *et al*., 2001; Iuliano, 2001; Rao & Balachandran 2002). Thus peroxynitrite, and not NO, has been proposed to be the ultimate cytotoxic species in many conditions acting through some mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of enzymes (e.g. MnSOD) and the depletion of glutathione. Moreover, peroxynitrite is also able to induce DNA damage (Inoue & Kawanishi, 1995; Salgo, 1995) resulting in inactivation of the nuclear enzyme PARS, in depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP) and lastly in cell death (Szabò, 1998). The realization of the cytotoxic potential of NO and peroxynitrite made it important to seek for pharmacological approaches, in order to neutralize NO and peroxynitrite-induced damage by inhibiting iNOS. The role of iNOS in pathologic condition have induced the development of selective iNOS inhibitors like GW274150 [(S)-2-amino-(1-iminoethylamino)-5-thioheptanoic acid]. This molecule is a novel NOS-inhibitor (sulphur-substituted acetamine amono acid), which acts in competition with L-arginine and has a very high degree of selectivity for iNOS when compared to either eNOS (>300-fold) or nNOS (>100-fold). In addition GW274150 is a long acting (5 h half-life in rats) iNOS inhibitor and is also able to inhibit LPS-mediated increase in plasma NO_2^- NO_3^- levels 14 h after single intraperitoneal dose (ED50 3 mg kg^{-1}). The compound N-3-aminomethyl-benzylacetamidine (1400W) also identified by Garvey (Garvey *et al*., 1997) has proved to be a further step forward, since it is not only highly selective as an iNOS inhibitor *versus* both eNOS and nNOS, but also penetrates cells and tissues. Inhibition of human iNOS by 1400W was competitive with L-arginine, nicotinamide adenine dinucleotide 3′-phosphate ($NADPH^+$)-dependent and developed relatively slowly, and no significant reversal of this inhibition was observed after 2 h. Efficacy and selectivity were maintained *in vivo* in the stringent test of its differential effects on vascular leakage; unlike L-NIO (L-N^5-(1-iminoethyl)-ornithine) and aminoguanidine, it suppressed the late, iNOS-driven phase of endotoxin-provoked leakage with no exacerbation of the early phase as it has been observed when eNOS and nNOS are inhibited (Garvey *et al*., 1997; Lazlo and Whittle, 1997). Unfortunately, this compound exhibits an acute toxicity at high doses, which is likely to prevent its safe therapeutic use in humans, but there is a significant therapeutic window such that it can be used as a pharmacological tool in a variety of animal models (Garvey *et al*., 1997; Lazlo and Whittle, 1997; Thomsen *et al*., 1997; Kankuri *et al*., 2001). Like with 1400W, the inhibition of iNOS activity caused by GW274150 is NADPH-dependent and develops very slowly, but is rapidly reversible and recent studies reports the role of this iNOS selective inhibitors in reducing organ injury in haemorrhagic shock, in collagen-induced arthritis and in renal ischaemia/reperfusion (Cuzzocrea *et al*., 2002; McDonald *et al*., 2002; Chatterjee *et al*., 2003). Therefore, the aim of this study was to investigate the role of GW274150 in a model of acute inflammation, carrageenan induced lung injury, and in order to get a better insight into the effect of GW274150 we investigated the results on: (1) intercellular adhesion molecule-1 (ICAM-1) expression (by immunohistochemistry), (2) pleural exudates and polymorphonuclear cells (PMNs) infiltration [myeloperoxidase (MPO) activity] (3) lipid peroxidation [malondialdehyde (MDA) levels], (4) IL-1β and TNF-α release (5) iNOS activity, (6) nitration of tyrosine residues (an indicator of the formation of peroxynitrite by immunohistochemistry), (7) PARS activation and (8) lung damage (histology).

Methods

Animals

Male Sprague–Dawley rats (150–200 g; Charles River, 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 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).

Experimental group

Rats were randomly allocated into the following groups: (i) CAR + vehicle group; rats were subjected to carrageenan-induced pleurisy and received the vehicle for GW274150 (saline solution) injected i.p. 5 min prior to carrageen ($n=10$), (ii) GW274150 group; same as the CAR + vehicle group but were administered GW274150 (2.5, 5 and 10 mg kg^{-1}) i.p. 5 min before carrageenan injection ($n=10$), (iii) Sham + saline group; sham-operated group in which identical surgical procedures to the CAR group was performed, except that the saline was administered instead of carrageenan ($n=10$). (iv) Sham + GW274150 group; identical to Sham + saline group except for the administration of GW274150 (2.5, 5 and 10 mg kg^{-1}) i.p. 5 min prior to identical surgical procedures ($n=10$).

Carrageenan-induced pleurisy

Rats were lightly anaesthetized under isoflurane and submitted to a skin incision at the level of the left sixth intercostals space. The underlying muscles were dissected and 0.2 ml saline alone or containing 1% (w v^{-1}) λ-carrageenan were injected into the pleural cavity. The skin incision was closed with a suture and the animals were allowed to recover. Rats were treated with GW274150 (2.5, 5 and 10 mg kg^{-1}) or with vehicle, both injected i.p. 5 min before carrageenan injection. At 4 h after the injection of carrageenan, the animals were killed under CO_2 vapour. The chest was carefully opened and the pleural cavity washed with 2 ml of saline solution with heparin (5 U ml^{-1}) and indomethacin (10 μg ml^{-1}). The exudates and the washing solution were removed by aspiration and the total volume measured. Exudates contaminated with blood were discarded. The results were calculated by subtracting the volume injected (2 ml) from the total volume recovered. The numbers of leucocytes in the exudates were suspended in phosphate buffer saline and counted with optical microscope by Burker's chamber after vital Trypan Blue stain.

Histological examination

For histopathological examination, biopsies of lungs were taken 4 h after the induction of pleurisy with carrageenan. The tissue slices were fixed in 10% neutral-buffered formaldehyde for 5 days, embedded in paraffin, and sectioned. The sections were stained with haematoxylin and eosin.

Myeloperoxidase activity

MPO activity, an index of PMNs accumulation, was determined as previously described (Mullane *et al*., 1985). Lung tissues, collected at the specified time, were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 $\times$ *g* at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured by spectrophotometry at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol ml^{-1} of peroxide at 37°C and was expressed in units g^{-1} of wet tissue.

Malondialdehyde levels

MDA levels in the lung tissue were determined as an indicator of lipid peroxidation as previously described (Ohkawa *et al*., 1979). Lung tissue collected at the specified time, was homogenized in 1.15% w v^{-1}) KCl solution. A 100 μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% (w v^{-1}) sodium dodecyl sulphate (SDS), 1.5 ml of 20% (v v^{-1}) acetic acid (pH 3.5), 1.5 ml of 0.8% (w v^{-1}) thiobarbituric acid and 600 μl distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 $\times$ *g* for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm. Levels of MDA are expressed as μM 100 mg^{-1} of wet tissue.

TNF-α release IL-1β

TNF-α and IL-1β levels were evaluated in the exudates at 4 h after the induction of pleurisy by carrageenan injection. The assay was carried out by using a colorimetric, commercial kit (Calbaiochem-Novabiochem Corporation, U.S.A.).

Measurement of nitrite–nitrate concentration in pleural exudates

Total nitrite in exudates, an indicator of NO synthesis, was measured as previously described (Cuzzocrea *et al*., 1998). Briefly, the nitrate in the sample was first reduced to nitrite by incubation with nitrate reductase (670 mU ml^{-1}) and 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^{-1}) naphthylethylendiamide dihydrochloride in H_2O and 1% (w v^{-1}) sulphanilamide in 5% (v v^{-1}) 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.

Determination of nitric oxide synthase activity

The calcium-independent conversion of L-arginine to L-citrulline in the homogenates of either pleural macrophages or lungs (obtained 4 h after carrageenan treatment in the presence or absence of GW274150) served as an indicator of iNOS activity (Cuzzocrea *et al*., 1998). Cells or tissues were homogenized on ice using a tissue homogenizer in a homogenation buffer composed of 50 mM Tris-HCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethylsulphonyl fluoride (PMSF) (pH 7.4). Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was measured in the homogenates as previously described (Cuzzocrea *et al*., 1998). Briefly, homogenates (30 μl) were incubated in the presence of [^{3}H]L-arginine (10 μM, 5 kBq per tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (2 mM) for 20 min at 22°C. Reactions were stopped by dilution with 0.5 ml of ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]L-citrulline activity was measured by a Beckman scintillation counter.

Immunohistochemical localization of ICAM-1, PARS and nitrotyrosine

ICAM-1, PARS and nitrotyrosine was detected in lung sections by immunohistochemistry. Tissues were fixed in 10% buffered formalin and 8 μm sections were prepared from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 min. Nonspecific adsorption was minimized by incubating the section in 2% normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (biotin

blocking kit, Vector Laboratories). The sections were then incubated overnight with (1) a mouse anti-rat antibody directed at ICAM-1 (1 : 500 in PBS, $v v^{-1}$) (DBA, Milan, Italy); (2) with a anti-PAR goat polyclonal antibody rat (1 : 500 in PBS, $v v^{-1}$) or (3) anti-nitrotyrosine antibody (1 : 1000 in PBS, $v v^{-1}$) (Upstate Biotech, Saranac Lake, NY, U.S.A.). Controls included buffer alone or nonspecific purified rabbit IgG. Sections were washed with PBS, and incubated with secondary antibody for 2 h at room temperature. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). The sections were stained with nuclear fast red. Immunocytochemistry photographs ($n = 5$) were assessed by densitometry as previously described (Cuzzocrea *et al*., 2000) by using Optilab Graftek software on a Macintosh personal computer.

Materials

Perchloric acid was obtained from Aldrich (Milan, Italy). Primary anti-nitrotyrosine antibody was from Upstate Biotech (DBA, Milan, Italy). All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, Milan, Italy).

Data analysis

All values in the figures and text are expressed as mean ± standard error of n observations, where n represents the number of animals studied. Data sets were examined by one-way analysis of variance and individual group means were then compared with Bonferroni or Student's unpaired t-test. A P-value less than 0.05 were considered significant. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days.

Results

Effect of GW274150 on carrageenan-induced pleurisy

At histological examinations lung sections carrageenan-treated rats showed oedema, tissue injury and extravasation of red cells as well as macrophage accumulation (Figure 1a, a1). GW274150 treatment reduced the degree of lung injury in a dose-related fashion (Figure 1b). No histological alteration and inflammatory cells infiltration were found in tissue sections from sham-operated rats (data not shown). All carrageenan-injected rats developed an acute pleurisy, producing turbid exudates (Figure 2a) that contained a large amount of PMNs (Figure 2b,b1). Oedema formation and PMNs infiltration in the pleural cavity were significantly attenuated in a dose-related manner by the intraperitoneal injection of GW274150 (Figure 2).

ICAM-1 expression and neutrophil infiltration are reduced in GW274150-treated rats

Assessment of neutrophil infiltration into the lung tissue was performed by measuring the activity of MPO, an enzyme contained into specific PMNs lysosomes. MPO activity and

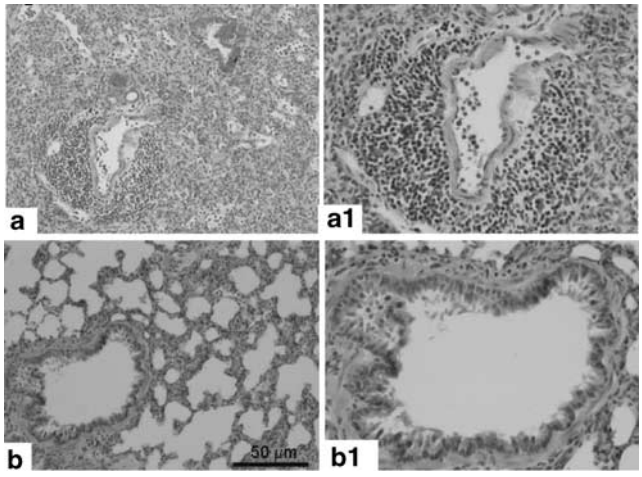

Figure 1 Lung section from a carrageenan-treated rats (a) demonstrating interstitial haemorrhage and PMNs accumulation (a1). Lung section from a carrageenan-treated rats after administration of GW214750 ($10\,mg\,kg^{-1}$) (b) demonstrating reduced interstitial haemorrhage and cellular infiltration. Original magnification: × 125. The figure is representative of at least three experiments performed on different experimental days.

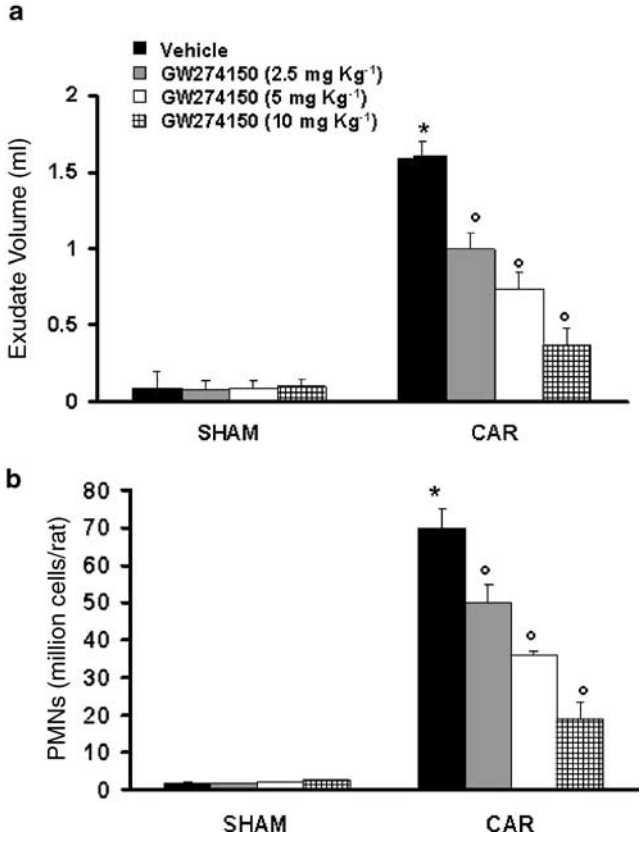

Figure 2 Volume exudate (a) and accumulation of PMNs (b) in pleural cavity at 4 h after carrageenan injection. GW214750 (2.5–5 and $10\,mg\,kg^{-1}$ i.p.) significantly reduced pleural exudation and leukocyte infiltration. Data are expressed as mean ± s.e.m. from $n = 10$ rats for each group. *$P < 0.01$ vs sham group; °$P < 0.01$ vs carrageenan.

MDA levels were significantly elevated after carrageenan injection (Figure 3a, b). The increase of the MPO activity was associated with the increase of immunohistochemical staining

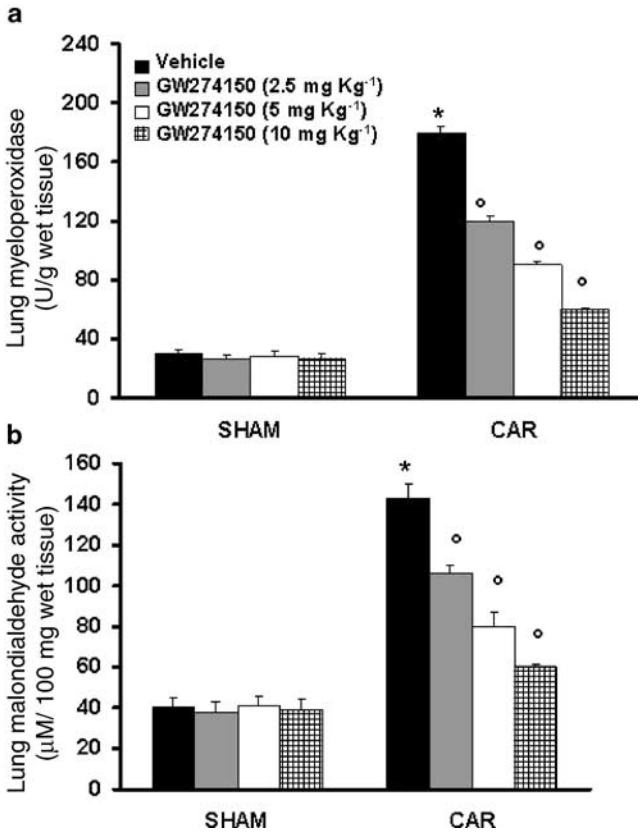

Figure 3 MPO activity (a) and MDA levels (b) in the lungs of carrageenan-treated rats killed after 4 h. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated rats in comparison to SHAM rats. GW214750 (2.5–5 and 10 mg kg^{-1} i.p.) reduced the carrageenan-induced increase in MPO activity and MDA levels. Data are expressed as mean ± s.e.m. from $n = 10$ rats for each group. *$P < 0.01$ vs sham group; °$P < 0.01$ vs carrageenan.

for ICAM-1 (Figures 4a, a1, 5) in the injured lung tissue. In rats treated with GW274150, tissue MPO activity and MDA levels (Figure 3a,b) were markedly reduced in comparison to those treated with vehicle. Significantly less positive staining for ICAM-1 was observed in the lung of GW274150-treated rats (Figures 4b, 5) and in the lung of sham-operated rats (Figure 5).

Effect of GW274150 on cytokine release

TNF-α and IL-1β levels resulted significantly increased 4 h after carrageenan injection in pleural exudates (Figure 6) and GW274150 treatment significantly attenuated in a dose-dependent manner both TNF-α and IL-1β levels (Figure 6).

GW274150 treatment reduces NO production

NO levels were also significantly increased in the exudate obtained from rats administered with carrageenan (Figure 7a). A significant increase in iNOS activity 4 h after administration of carrageenan was detected in lungs obtained from rats subjected to carrageenan-induced pleurisy (Figure 7b). GW274150 treatment is able to significantly attenuate both NO levels and iNOS activity in a dose-related manner (Figure 7).

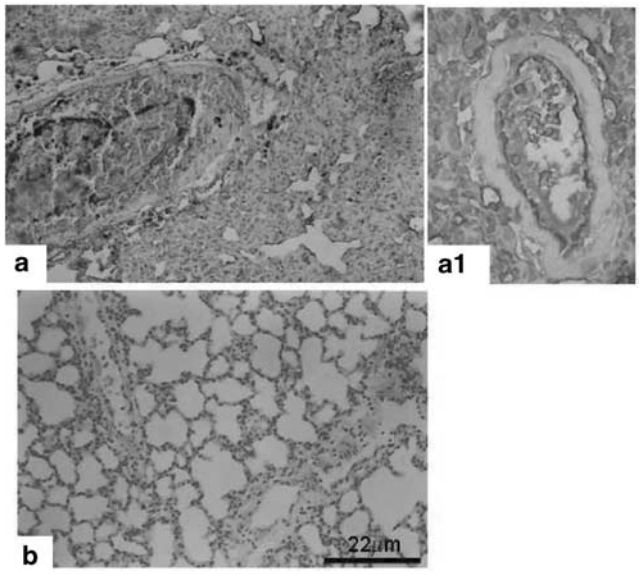

Figure 4 Immunohistochemical analysis of ICAM-1 expression. A positive staining was observed 4 h after carrageenan injection (a) localized mainly around the vessels (a1). In the lungs of GW214750 (10 mg kg^{-1})-treated rats no positive staining was observed (b). The figure is representative of at least three experiments performed on different experimental days.

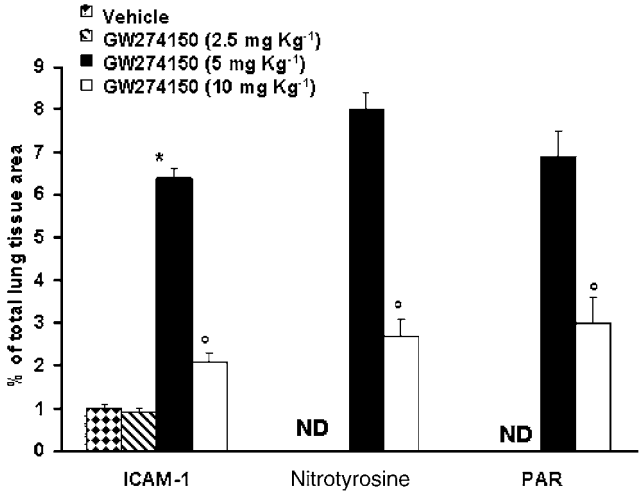

Figure 5 Typical densitometry evaluation. Densitometry analysis of immunocytochemistry photographs ($n = 5$) for ICAM-1, nitrotyrosine and PAR from lung was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as percent of total tissue area. *$P < 0.01$ vs Sham. °$P < 0.01$ vs IR.

GW274150 treatment reduced nitrotyrosine and PARS formation

Lungs of carrageenan-treated rats were characterized by a positive staining for nitrotyrosine, a marker of nitrosative injury (Figures 5, 8a,a1). No positive nitrotyrosine staining was found in lung tissue of rats treated with GW274150 (Figures 5, 8b). Lung sections were also taken in order to determine the immunohistological staining for poly ADP-ribosylated (PAR) proteins (an indicator of PARS activation). Immunohistochemical analysis of lung section obtained from

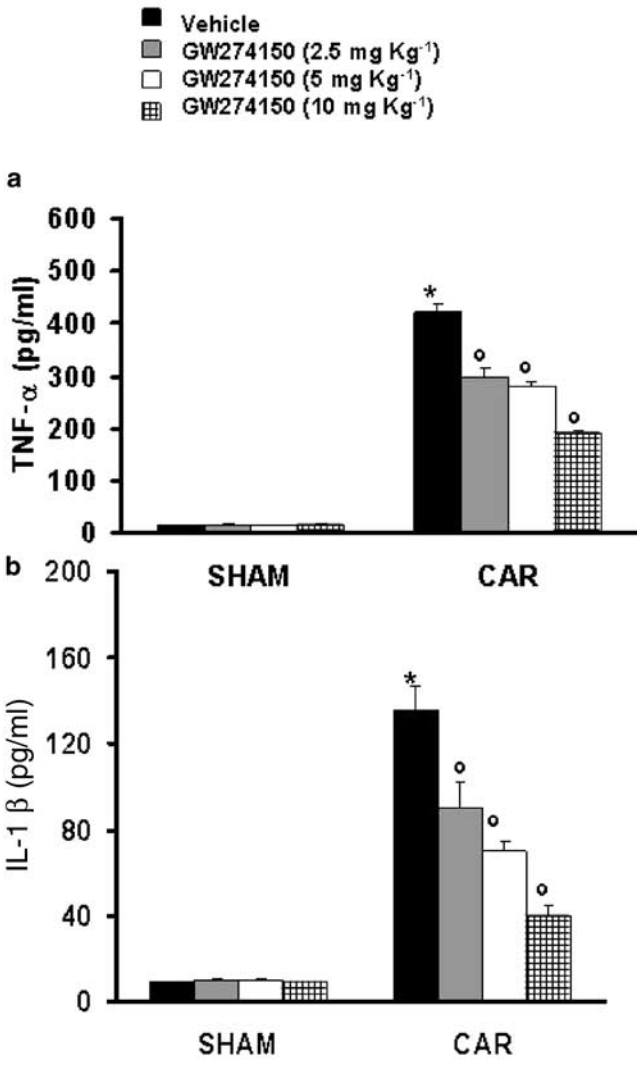

Figure 6 Exudate levels of TNF-α (a) and IL-1β. GW214750 (2.5–5 and 10 mg kg^{-1} i.p.) significantly reduced carrageenan-induced proinflammatory cytokines production. Data are expressed as mean$\pm$s.e.m. from $n=10$ rats for each group. *$P<0.01$ vs sham group; $^{\circ}P<0.01$ vs carrageenan.

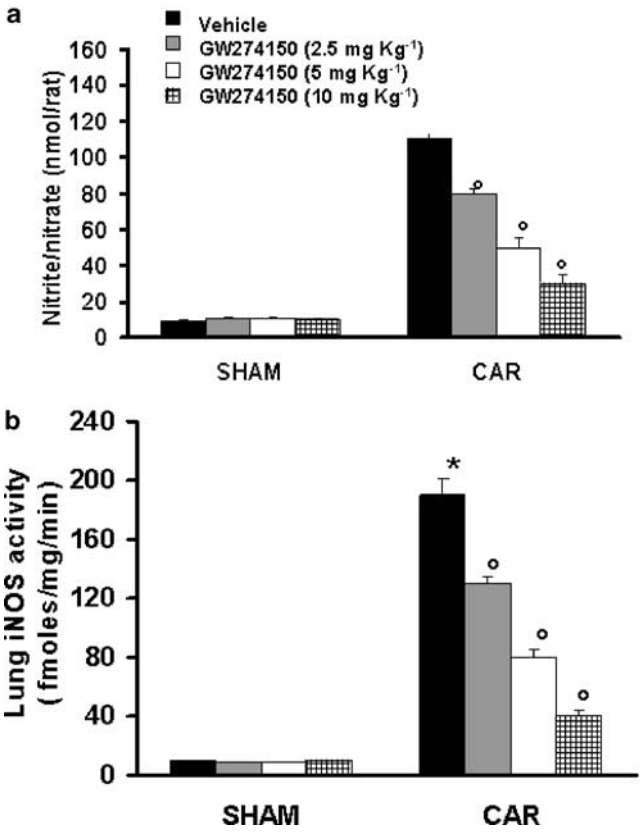

Figure 7 Nitrite and nitrate concentrations in pleural exudates (a) and iNOS activity (b). Nitrite and nitrate levels and iNOS activity in carrageenan-treated rats were significantly increased *versus* sham group. GW214750 (2.5–5 and 10 mg kg^{-1} i.p.) significantly reduced the carrageenan-induced increase of nitrite and nitrate levels and the iNOS activity. Data are expressed as mean$\pm$s.e.m. from $n=10$ rats for each group. *$P<0.01$ vs sham group; $^{\circ}P<0.01$ vs carrageenan.

rats subjected to carrageenan-induced pleurisy revealed a positive staining for PAR (Figures 5, 9a,a1). In contrast, significantly less positive PAR staining was found in the lungs of rats treated with GW274150 and also subjected to carrageenan-induced pleurisy (Figures 5, 9b). It is important to underline that there was no staining for either nitrotyrosine or PAR in lungs obtained from sham-operated rats (Figure 5).

Discussion

The inflammatory process is invariably characterized by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and by a release of chemicals from tissues and migrating cells (Vane & Botting, 1987; Tomlinson *et al*., 1994). Carrageenan-induced local inflammation is commonly used to evaluate nonsteroidal anti-inflammatory drugs (NSAID). Therefore, carrageenan-induced local inflammation (paw oedema or pleurisy) is a useful model to assess the contribution of mediators involved in vascular changes associated with acute inflammation (Di Rosa & Willoughby, 1971). In particular, the initial phase of inflammation (oedema, 0–1 h), which is not inhibited by NSAID such as indomethacin or aspirin, has been attributed to the release of histamine, 5-hydroxytryptamine and bradykinin, followed by a late phase (1–6 h) mainly sustained by prostaglandin release (Di Rosa & Willoughby, 1971). It appears that the onset of the carrageenan local 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 (Dawson *et al*., 1991; Cuzzocrea *et al*., 1997).

Furthermore, there is a large amount of evidence that the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contribute to tissue damage (Salvemini *et al*., 1996a). Inhibitors of NOS activity reduce the development of carrageenan-induced inflammation and support a role for NO in the pathophysiology associated with this model of inflammation and, in addition to NO, peroxynitrite is also generated in carrageenan-induced inflammation (Wei *et al*., 1995; Cuzzocrea *et al*., 1997).

Thus NO, produced by iNOS, plays a pivotal role during inflammatory processes and iNOS selective inhibitors seems to

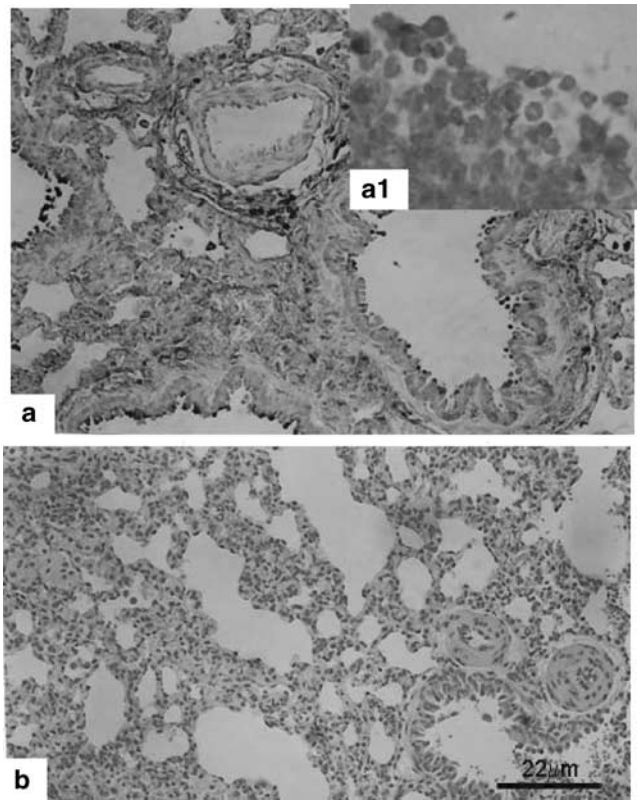

Figure 8 Immunohistochemical analysis of nitrotyrosine formation. A positive staining was observed 4 h after carrageenan injection (a) localized mainly to macrophages and some epithelial cells (a1). In the lungs of GW214750 (10 mg kg^{-1})-treated rats no positive staining was observed (b). The figure is representative of at least three experiments performed on different experimental days.

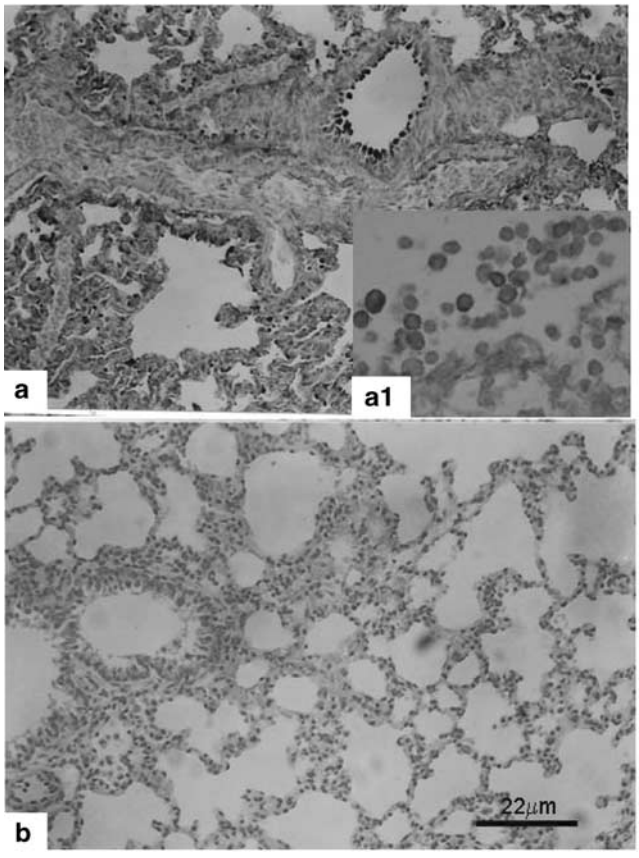

Figure 9 Immunohistochemical analysis of PARS activation. A positive staining was observed 4 h after carrageenan injection (a) localized mainly to macrophages and some epithelial cells (a1). In the lungs of GW214750 (10 mg kg^{-1})-treated rats no positive staining was observed (b). The figure is representative of at least three experiments performed on different experimental days.

be very useful in interfering with this NO production. The role of iNOS-derived NO, or peroxynitrite, as an amplifier of the inflammatory response is now also supported by previous observations: inhibition of iNOS suppresses TNF-α production in the delayed phase of allergic encephalomyelitis (Anggard, 1991); inhibition of iNOS suppresses IL-1, collagen and stromelysin production in arthritis (Parker, 1987); inhibition of iNOS suppresses INF-γ production in a murine model of leishmaniasis (Stamler and Loscalzo, 1991) and the expression of certain chemokines is suppressed in the absence of iNOS in zymosan-induced peritonitis (Davidge *et al*., 1995). In addition it has been previously demonstrated that interventions which reduce the generation or the effects of NO exert beneficial effects in a variety of models of inflammation, including the carrageenan-induced pleurisy model used here (Salvemini *et al*., 1996b), and it has also been demonstrated that therapeutic interventions include melatonin (Cuzzocrea *et al*., 1997) and a vitamin E-like antioxidant (Cuzzocrea *et al*., 1999).

GW274150 is a novel, potent and selective inhibitor of iNOS activity and previous studies have demonstrated its protective effect in organ injury in haemorrhagic shock, in renal ischaemia and reperfusion and in a model of collagen-induced arthritis (Cuzzocrea *et al*., 2002; McDonald *et al*., 2002; Chatterjee *et al*., 2003).

This study provides the first evidence of the protective role of GW274150 in an acute model of inflammation, carrageenan-induced lung injury. Our results demonstrate that GW274150 attenuates: (i) the development of carrageenan-induced pleurisy, (ii) ICAM-1 expression, (iii) the infiltration of the lung with PMNs, (iv) the release of proinflammatory cytokine, (v) the activity of iNOS, (vi) nitrotyrosine and PARS activation and (vii) the degree of lung injury caused by injection of carrageenan. All of these findings support the view that GW274150 attenuates the degree of acute inflammation in the rat.

There are evidences that proinflammatory cytokines as TNF-α and IL-1β help to propagate the extension of a local or systemic inflammatory process (McDonald *et al*., 2002) We confirm here that the inflammatory process (carrageenan-induced pleurisy) leads to a substantial increase in the levels of TNF-α and IL-1β in the exudates which likely contribute in different capacities to the evolution of acute inflammation.

A number of recent studies have also demonstrated that the recruitment of cells into an area of inflammation may be mediated not only by C5a, leukotrienes, PAF, or bacterial-derived peptides, but also by a novel group of small proteins with relatively specific chemotactic activity for leukocyte subpopulations (e.g. NO). Endothelial cells appear to be major regulators of the neutrophil traffic, regulating the process of neutrophil chemoattraction, adhesion and migration from the vasculature to the tissue. During the inflamma-

tory process ICAM-1, constitutively expressed on the surface of endothelial cells, is then involved in the neutrophil adhesion (Cuzzocrea *et al*., 2002; Chatterjee *et al*., 2003). Hypoxic endothelial cells synthesize proinflammatory cytokines, which can upregulate endothelial expression of the constitutive adhesion molecule ICAM-1 in autocrine fashion (Mannel & Echtenacher, 2000). In the present study it is reported that acute inflammation in the rat results in a significant infiltration of inflammatory cells in the pleural cavity as well as in lung tissue and we demonstrated that treatment with GW274150 reduces this inflammatory cells infiltration as assessed by proinflammatory cytokine production (TNF-α and IL-1β), by ICAM-1 expression, by the specific granulocyte enzyme MPO and with the moderation of the tissue damage as evaluated by histological examination. Neutrophils are recruited into the tissue by local production of cytokines and can then contribute to tissue destruction by the production of reactive oxygen metabolites, granule enzymes and cytokines that further amplify the inflammatory response by their effects on macrophages and lymphocytes (Lawrence & Spinger, 1991).

An intense immunostaining of nitrotyrosine formation also suggested that a structural alteration of the lung had occurred, most probably due to the formation of highly reactive nitrogen derivatives. Recent evidence indicates, in fact, that several chemical reactions, involving nitrite, peroxynitrite, hypochlorous acid and peroxidases can induce tyrosine nitration and may contribute to tissue damage (Shreeniwas *et al*., 1992; Farhood *et al*., 1995). There is a large amount of evidence that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage (Eiserich *et al*., 1996; Salvemini *et al*., 2001). In addition to NO, peroxynitrite is also generated in carrageenan-induced acute inflammation (Salvemini *et al*., 1996b).

Therefore, in this study we clearly demonstrate that GW274150 treatment prevent the induction of iNOS and the formation of peroxynitrite. ROS 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 and its depletion leads to a rapid fall in intracellular ATP. This process has been termed 'the PARS suicide hypothesis' (Salvemini *et al*., 1998). There is recent evidence that the activation of PARS may also play an important role in inflammation (Holmdahl *et al*., 1985; Szabò *et al*., 1997; Salvemini *et al*., 1998) and we demonstrate here that GW274150 treatment reduced the activation of PARS during carrageenan-induced pleurisy in the lung.

In conclusion, this study provides the first evidence that GW274150 causes a substantial reduction of acute inflammation in the rat. Furthermore, our data afford evidence that GW274150 reduces proinflammatory cytokines release, the degree of oxidative stress and of PARS activation during carrageenan-induced pleurisy. Thus, we demonstrate here that the mechanisms underlying the protective effects of GW274150 are dependent by a reduction of (i) iNOS activity and the nitration of proteins by peroxynitrite, (ii) the formation of the proinflammatory cytokines, and (iii) the inflammatory cells infiltration.

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