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Effect of methylguanidine in carrageenan-induced acute inflammation in the rats

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Abstract

In vitro and in vivo studies have demonstrated that methylguanidine, an inhibitor of nitric oxide synthase (NOS), is also able to reduce tumour necrosis factor-α (TNF-α) release. In the present study, we evaluated the anti-inflammatory potential of methylguanidine treatment in two models of acute inflammation (carrageenan-induced paw edema and pleurisy) where oxyradical, nitric oxide (NO) and prostaglandins play a crucial role in the inflammatory processes. Our data show that methylguanidine, given intraperitoneally at the dose of 30 mg/kg, inhibits the inflammatory response reducing significantly ($P<0.05$) paw swelling, pleural exudates formation, mononuclear cell infiltration and histological injury. Furthermore, our data suggests that there is a significant ($P<0.05$) reduction in the activity and expression both of the inducible NOS (iNOS) and of cyclooxygenase-2 in lung tissue of pleurisy model. Methylguanidine is also able to reduce the appearance of nitrotyrosine and of the nuclear enzyme poly(adenosine diphosphate [ADP]-ribose) synthase immunoreactivity in the inflamed lung tissues. Treatment with aminoguanidine, the reference drug, significantly reduced all the evaluated pro-inflammatory parameters in carrageenen-treated rats. Taken together, the present results demonstrate that methylguanidine exerts potent anti-inflammatory effects that could be, in part, related to an inhibition of the expression/activity of the iNOS and cyclooxygenase-2 and, another part, may be related to a reduction of TNF-α release.

Keywords: Methylguanidine; Carrageenan-induced inflammation; TNF-α (tumor necrosis factor-α); Reactive oxygen specie; iNOS (nitric oxide (NO) synthase, inducible); Cyclooxygenase-2

1. Introduction

Nitric oxide (NO) is a pleiotropic mediator which acts in a variety of physiological and pathophysiological processes (Nathan, 1992; Dinerman, 1993; Szabò, 1995; Southan and Szabò, 1996). NO is produced from the oxidation of L-arginine by the enzyme NO synthase (Moncada and Palmer, 1991; Moncada and Higgs, 1993), which occurs in two major classes one is constitutive (including endothelial and neuronal isoforms) and another is inducible (including macrophagic isoform). The constitutive NO synthase (cNOS) is Ca^{2+}-dependent, releases NO under physiological condition in various cells, including endothelial cells and neurons, and NO released by this isoform is involved in the regulation of blood pressure, organ blood flow distribution and the inhibition of the adhesion and activation of platelets and polymorphonuclear granulocytes. The inducible isoform of NOS (iNOS) is Ca^{2+}-independent and can be induced by pro-inflammatory agents, such as endotoxins (bacterial lipopolysaccharide), interleukin-1β, tumor necrosis factor-α (TNF-α) and interferon-γ, in endothelial and smooth-muscle cells, in macrophages and in other cell types (Nathan, 1992; Dinerman, 1993; Szabò, 1995; Southan and Szabò, 1996; Moncada and Palmer, 1991). Enhanced formation NO following the induction of iNOS has been implicated in the pathogenesis of shock and inflammation (Nathan, 1992). Recently, it has been suggested that some of the cytotoxic effects of NO are tightly related to the production of

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peroxynitrite, a high-energy oxidant deriving by the rapid reaction of NO with superoxide (Crow and Beckmann, 1995; Pryor and Squadrito, 1995; Beckman et al., 1990).

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 (Rao and Balachandran, 2002; Iuliano, 2001; Yamada and Grisham, 1991; Cuzzocrea et al., 2001). Some authors proposed the involvement of peroxynitrite in mediating cell damage during various pathophysiological conditions like inflammation or oxidant stress (Cuzzocrea et al., 2001; Salvemini et al., 1996a). Peroxynitrite, and not NO, has been in fact proposed to be the ultimate cytotoxic species in many conditions acting trough some mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of enzymes (e.g. manganese superoxide dismutase) and the depletion of glutathione. Moreover, peroxynitrite are also able to induce DNA damage (Inoue and Kawanishi, 1995; Salgo, 1995) resulting in inactivation of the nuclear enzyme poly(adenosine diphosphate [ADP]-ribose) synthase, in depletion of nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP) and lastly in cell death (Szabò, 1998).

Thus, the realization of the cytotoxic potential of nitric oxide and peroxynitrite made it important to seek for pharmacological approaches in order to neutralize nitric oxide and peroxynitrite-induced damage. These efforts have resulted in significant advances over the last years. A new class of iNOS inhibitors has been developed by the guanidine group of the amino acid L-arginine, such as guanidine, aminoguanidine, mercaptoethylguanidine and methylguanidine, which is well known to be a product of protein catabolism (Southan and Szabò, 1996; Zhang et al., 2001; MacAllister and Whitley, 1994). We have previously demonstrated that methylguanidine attenuates NO production by inhibiting both constitutive and inducible NOS (Sorrentino et al., 1997).

In the present study, we have investigated the effects of methylguanidine on rat paw edema and lung injury associated with carrageenan injection. In particular, we have investigated the effect of methylguanidine on (1) paw edema development, (2) polymorphonuclear leukocyte infiltration (myeloperoxidase activity), (3) lipid peroxidation (malondialdehyde levels), (4) iNOS and cyclooxygenase-2 expression (by Western blot analysis), (5) nitration of tyrosine residues (an indicator of the formation of peroxynitrite by immunohistochemistry), (6) poly(ADP-ribose) synthase activation and (7) lung damage (histology).

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (150–200 g; Charles River, Milan, Italy, used for the pleurisy studies) and Wistar rats (150–200 g; Harlan, Milan; Italy, used for paw edema) 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).

2.2. Carrageenan-induced paw edema

Rats were lightly anaesthetized under isofluorane and received a subplantar injection 0.1-ml saline containing 1% w/v λ-carrageenan into the right hind paw. Methylguanidine (30 mg/kg), dissolved in saline solution, was given intraperitoneally (i.p.) in a total volume of 1 ml/kg starting 1 h before and at 1 and 3 h after carrageenan injection. Sham-operated group of animals received the same volume of vehicle at the same time. The volume of the paw was measured by an hydroplethysmometer (Ugo Basile, Milan, Italy) immediately after the injection as previously described (Gerold et al., 1966). Subsequent readings of the volume of the same paw were carried out for 6 h at 60-min intervals and compared to the initial readings. In a separated set of experiments, animals were treated with aminoguanidine (100 mg/kg), as a reference drug, 1 h before carrageenan administration. The doses of methylguanidine and aminoguanidine used here have previously been reported to reduce lipopolysaccharide -induced shock (Autore et al., 1999; Vona-Davis et al., 2002).

2.3. Carrageenan-induced pleurisy

Rats were lightly anaesthetized under isofluorane and submitted to a skin incision at the level of the left sixth intercostal space. The underlying muscles were dissected and 0.2-ml saline alone or containing 1% (w/v) 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 methylguanidine (30 mg/kg) or with vehicle (sham-operated group), all injected i.p. starting 1 h before and at 1 and 3 h after carrageenan injection. At 4 h after the injection of carrageenan, the animals were sacrificed 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) and indomethacin (10 μg/ml). The exudate was 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 number of leucocytes in the exudate was suspended in phosphate buffer saline and counted with optical microscope by Burker's chamber after vital Trypan Blue stain. In a separated set of experiments animals were treated with aminoguanidine (100 mg/kg), as a reference drug, 1 h before carrageenan administration. The doses of methylguanidine and aminoguanidine used here

have previously been reported to reduce lipopolysaccharide-induced shock (Autore et al., 1999; Vona-Davis et al., 2002). It is important to point out that the peak serum levels of methylguanidine administered i.p. in normal rats is obtained at 1 h after the injection and at 6 h after the serum levels is less the half of the peak obtained at 1 h (Yokozawa et al., 1989).

2.4. Measurement of arterial blood pressure indirectly in conscious rat

Mean arterial blood pressure in conscious rats was measured by a Blood Pressure Recorded (UGO BASILE, Biological Research Apparatus, 21025 Comerio, Italy) using a technique described by Gerold (Gerold et al., 1966). Briefly, rats were divided in three groups: sham-operated, methylguanidine- and aminoguanidine-treated rats, and then treated as described above. After a 1-week training period, rats were treated and blood pressure was measured before and after treatments (30 min after the last administration). A tail cuff, consistently about 2 cm from the base of the tail was placed and arterial blood pressure was measured. Heart rate was detected by a pulse rate counter placed after the tail cuff.

2.5. Histological examination

Lung biopsies were taken 4 h after injection of carrageenan. Lung biopsies were fixed for 1 week in 10% (w/v) phosphate buffer saline (PBS)-buffered formaldehyde solution at room temperature, dehydrated using graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, USA). Sections were then deparaffinized with xylene, stained with hematoxylin and eosin. All sections were studied using light microscopy (Dialux 22 Leitz).

2.6. Myeloperoxidase activity

Myeloperoxidase activity, an index of polymorphonuclear leukocyte 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 × *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. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37 °C and was expressed in units/g of wet tissue.

2.7. Malondialdehyde levels

Malondialdehyde 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 homogenised in 1.15% (w/v) KCl solution. A 100-μl aliquot of the homogenate was added to a reaction mixture containing 200 μl of 8.1% (w/v) sodium dodecyl sulfate (SDS), 1.5 ml of 20% (v/v) acetic acid (pH 3.5), 1.5 ml of 0.8% (w/v) thiobarbituric acid and 600 μl distilled water. Samples were then boiled for 1 h at 95 °C and centrifuged at 3000 × *g* for 10 min. The absorbance of the supernatant was measured using spectrophotometry at 650 nm. Levels of malondialdehyde are expressed as μM/100 mg of wet tissue.

2.8. TNF-α release

TNF-α release in the pleural exudates was measured by enzyme immunoassay according to the manufacturer's instruction (Cayman Chemical) and TNF-α levels are expressed as U/ml.

2.9. Measurement of nitrite–nitrate concentration in pleural exudate

Total nitrite in exudates, an indicator of NO synthesis, was measured as previously described (Cuzzocrea et al., 1998a). Briefly, the nitrate in the sample was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and â-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) naphthylethylenediamide 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 a 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.

2.10. Measurement of prostaglandin E_2 levels in the exudate

The amount of prostaglandin E_2 in the pleural exudates was measured by enzyme immunoassay according to the manufacturer's instruction (Cayman Chemical). Prostaglandin E_2 levels are expressed as pg/rat.

2.11. Western blot analysis for iNOS and cyclooxygenase-2 expression

Lung tissue were homogenated in a buffer containing: HEPES 20 mM, $MgCl_2$ 1.5 mM, NaCl 0.4 mM, EDTA 1 mM, EGTA 1 mM, dithiothreitol 1 mM, phenyl methyl sulphonyl fluoride 0.5 mM, trypsin inhibitor 15 μg/ml, pepstatin 3 μg/ml, leupeptin 2 μg/ml, benzidamin 40 μM, nonidet P-40 1% and glycerol 20%. Protein concentration was estimated by the Bio-Rad protein assay using bovine serum albumine as standard. Equal amounts of protein (70

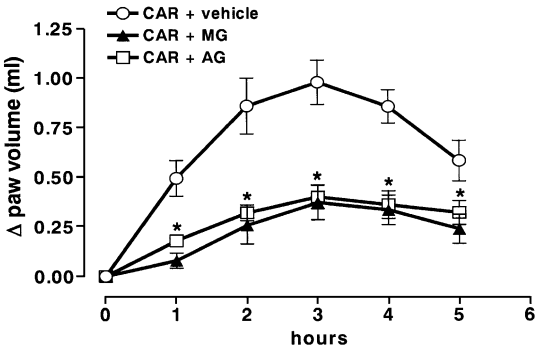

Fig. 1. Effect of methylguanidine (30 mg/kg i.p.) on rat paw edema development elicited by carrageenan in the rat. Methylguanidine (30 mg/kg i.p.) and aminoguanidine (100 mg/kg) significantly inhibited rat paw edema formation at the indicated time points. Data are means of mean ± S.E.M. from $n = 10$ rats for each group. *$P < 0.01$ vs. carrageenan-induced edema formation at the indicated time points.

μg) were dissolved in Laemmli's sample buffer, boiled and run on a sodium dodecyl sulphate-polyacrylamide gel electrophoresis minigel (8% polyacrylamide) and then transferred for 40 min at 5 mA/cm^2 into a 0.45-μm hybond polyvinylidene difluoride membrane. Membranes were blocked for 40 min in PBS and 5% (w/v) non-fat milk and subsequently probed overnight at 4 °C with mouse monoclonal anti-iNOS (1:10,000) or anti-cyclooxygenase-2 (1:500) antibodies (in PBS, 5% w/v non-fat milk and 0.1% Tween-20). Blots were then incubated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (IgG; 1:5.000) for 1 h at room temperature. Immunoreactive bands were visualized using electrochemiluminescence assay detection system according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of iNOS and cyclooxygenase-2 on X Omat films were quantified by scanning densitometry (Imaging Densitometer GS-700 BIO-RAD USA).

2.12. Immunohistochemical localization of nitrotyrosine and poly(ADP-ribose) synthase

Tyrosine nitration, a specific "footprint" of peroxynitrite formation, was detected as previously described (Cuzzocrea et al., 1998b) in lung sections by immunohistochemistry. At the specified time following the carrageenan injection, 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. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in phosphate-buffered saline 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 1:1000 dilution of primary anti-nitrotyrosine antibody (Upstate Biotech, Saranac Lake, NY) or with control solutions. Controls included buffer alone or non-specific purified rabbit IgG. Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories).

2.13. Data analysis

All values in the figures and text are expressed as mean ± standard error of the mean of n observations, where n represents the number of animals studied. Data sets were examined by one- and two-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 was considered significant. In the experiments involving histology or immunohistochemistry, the figures shown are representative of at least three experiments performed on different experimental days.

3. Results

Before edema, mean arterial blood pressure measurement of vehicle- and methylguanidine-treated rats was evaluated. Treatment of rats with methylguanidine (30 mg/kg i.p.), as described above, did not modify rat blood pressure, indi-

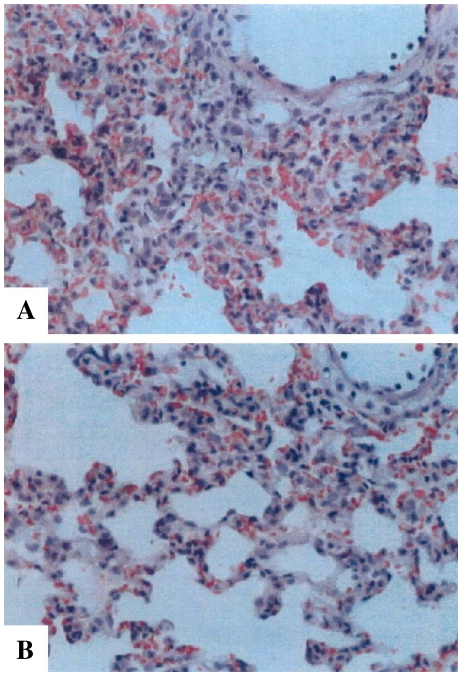

Fig. 2. Effect of methylguanidine on lung injury. Lung section from a carrageenan-treated rats (A) demonstrating interstitial hemorrhage and polymorphonuclear neutrophil accumulation. Lung section from a carrageenan-treated rats after administration of methylguanidine (30 mg/kg) (B) demonstrating reduced interstitial hemorrhage and cellular infiltration. Original magnification: × 125. Figure is representative of at least three experiments performed on different experimental days.

cating a lack effect on constitutive, endothelial NO production (sham-operated group 104.22 ± 5.94 mm Hg, methylguanidine-treated group 104.0 ± 3.56 mm Hg, $n = 6$).

3.1. Effects of MG on the course of the carrageenan-induced paw edema

Intraplantar injection of carrageenan in rats led to a time-dependent increase in paw volume that was maximal after 3 h (Fig. 1). The maximal increase in rat paw volume was observed 3 h after carrageenan administration (maximal in paw volume: 1.21 ± 0.08 ml). However, carrageenan-induced paw edema was significantly reduced by treatment with methylguanidine and aminoguanidine at every hour (62.0% and 59.1%, respectively, $P < 0.01$; Fig. 1).

3.2. Effects of MG in carrageenan-induced pleurisy

Histological examination of lung sections revealed a significant tissue damage in carrageenan-treated rats (Fig. 2). Lung sections of carrageenan-treated rats showed edema, tissue injury and extravasation of red cells as well as macrophage accumulation (Fig. 2A). Methylguanidine (Fig. 2B) and aminoguanidine (data not shown) treatment reduced the degree of lung injury.

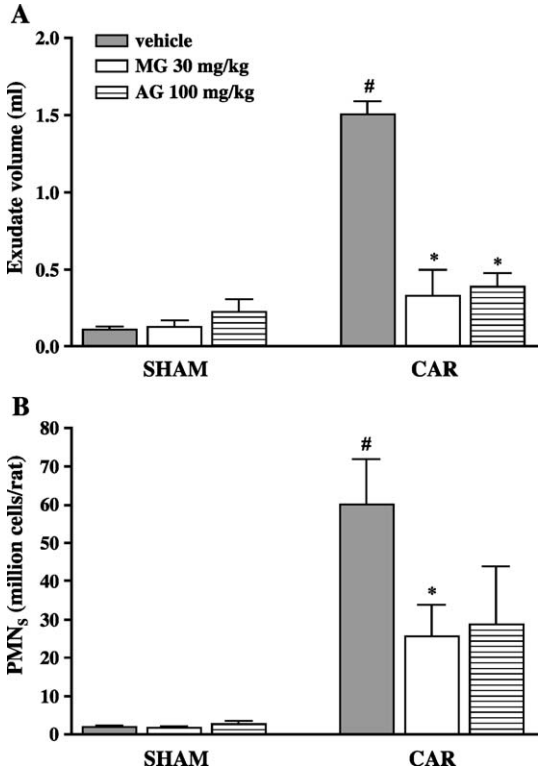

Fig. 3. Effect of methylguanidine on carrageenan-induced inflammation. Volume exudate (A) and accumulation of polymorphonuclear cells (B) in pleural cavity at 4 h after carrageenan injection. Methylguanidine (30 mg/kg i.p.) and aminoguanidine (100 mg/kg) significantly reduced pleural exudation and leukocyte infiltration. Data are means of mean ± S.E.M. from $n = 10$ rats for each group. $^{*}P < 0.01$ vs. carrageenan. $^{\#}P < 0.01$ vs. sham-operated group.

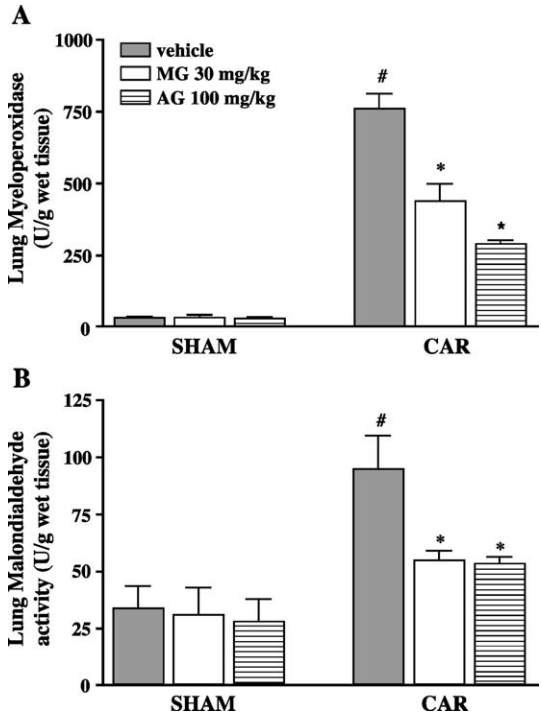

Fig. 4. Effect of methylguanidine on myeloperoxidase activity and malondialdehyde levels in the lung. Myeloperoxidase activity (A) and malondialdehyde levels (B) in the lungs of carrageenan-treated rats sacrified after 4 h. Malondialdehyde levels and myeloperoxidase activity were significantly increased in the lungs of the carrageenan-treated rats in comparison to sham-operated rats. Methylguanidine (30 mg/kg i.p.) and aminoguanidine (100 mg/kg) reduced the carrageenan-induced increase in malondialdehyde levels and myeloperoxidase activity. Data are means of mean ± S.E.M. from $n = 10$ rats for each group. $^{*}P < 0.01$ vs. carrageenan. $^{\#}P < 0.01$ vs. sham-operated group.

All carrageenan-injected rats developed an acute pleurisy, producing 1.51 ± 0.08 ml/rat of turbid exudate (Fig. 3A) that contained a large amount of polymorphonuclear leukocyte (60 ± 12 × 10^6/rat; Fig. 3B). Neutrophils also infiltrated the lung tissue (Fig. 4A) and this infiltration was

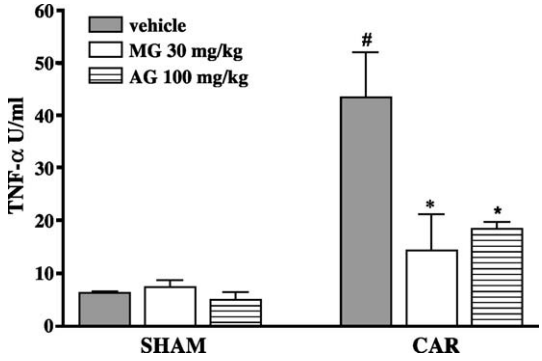

Fig. 5. Exudate levels of TNF-α. Methylguanidine (30 mg/kg i.p.) and aminoguanidine (100 mg/kg) significantly reduced carrageenan-induced elevation. Data are means of mean ± S.E.M. from $n = 10$ rats for each group. $^{*}P < 0.01$ vs. carrageenan. $^{\#}P < 0.01$ vs. sham-operated group.

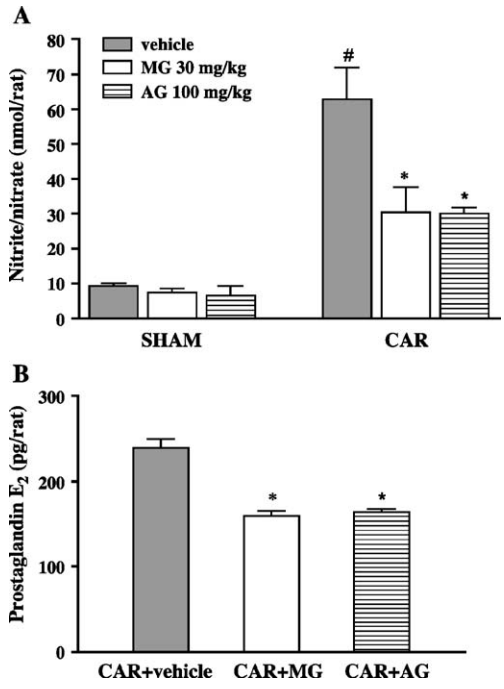

Fig. 6. Nitrite and nitrate concentrations (A) and prostaglandin levels in pleural exudate (B) at 4 h after carrageenan administration. Nitrite and nitrate and prostaglandin levels in carrageenan-treated rats were significantly increased vs. sham-operated group. Methylguanidine (30 mg/kg i.p.) and aminoguanidine (100 mg/kg) significantly reduced the carrageenan-induced elevation of nitrite and nitrate and prostaglandin levels. Data are means of mean ± S.E.M. from $n=10$ rats for each group. $^{*}P<0.01$ vs. carrageenan. $^{\#}P<0.01$ vs. sham-operated group.

associated with lipid peroxidation as evidenced by an increase in malondialdehyde levels (Fig. 4B). Methylguanidine and aminoguanidine treatment significantly reduced edema formation (78.0% and 74.0%, respectively) polymorphonuclear leukocyte infiltration in the pleural cavity (57.1% and 51.9%, respectively), neutrophil infiltration (42.5% and 61.9%, respectively) and lipid peroxidation in lung tissue (42.1% and 43.8%, respectively; Figs. 3–4).

TNF-α release in pleural exudates resulted augmented 4 h after carrageenan injection (43.57 ± 8.56 U/ml, $P<0.05$ compared to sham-operated group); methylguanidine and aminoguanidine treatment significantly attenuated these levels (14.48 ± 6.92 and 18.5 ± 1.35 U/ml, respectively, $P<0.01$) (Fig. 5).

A significant increase in iNOS expression 4 h after carrageenan injection, assayed by Western blot analysis, was detected in lungs obtained from rats subjected to carrageenan-induced pleurisy (Fig. 8A,B) and NO levels were also significantly ($P<0.01$) increased in the pleural exudate after carrageenan challenge (Fig. 6A). Methylguanidine and aminoguanidine treatment are able to significantly attenuate NO levels (51.4%; Fig. 6A) and methylguanidine is also able to attenuate iNOS expression (38.0%; Fig. 8A,B).

Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan also revealed a positive staining for nitrotyrosine (Fig. 7A). In contrast, no positive staining for nitrotyrosine was found in the lungs of rats treated with methylguanidine (Fig. 7B) or with aminogua-

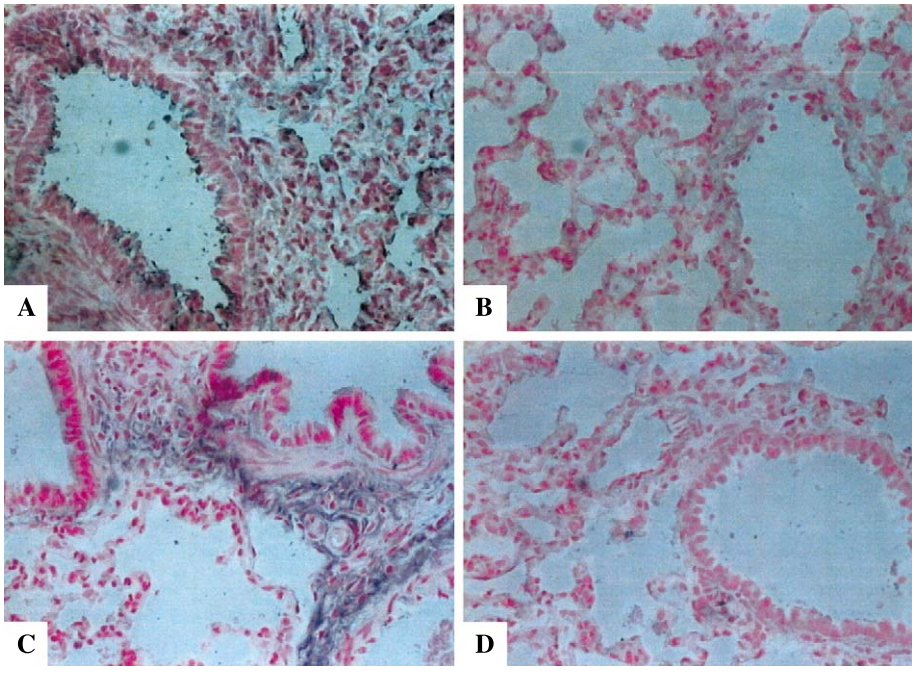

Fig. 7. Effect of methylguanidine on nitrotyrosine and poly(ADP-ribose) synthase activation formation. Four hours after carrageenan injection, positive staining for nitrotyrosine (A) and poly(ADP-ribose) synthase was observed (C). There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats treated with methylguanidine (30 mg/kg i.p.) was observed for nitrotyrosine (B) and poly(ADP-ribose) synthase (D). Original magnification: × 150. This figure is representative of at least three experiments performed on different experimental days.

nidine (data not shown). Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan also revealed a positive staining for poly(ADP-ribose) synthase (Fig. 7C) and no staining for poly(ADP-ribose) synthase was found in the lungs of carrageenan-treated rats treated with methylguanidine (Fig. 7D) or with aminoguanidine (data not shown). There was no staining for either nitrotyrosine or poly(ADP-ribose) synthase in lungs obtained from the sham-operated group of rats (data not shown).

Cyclooxygenase-2 activity in carrageenan-induced pleurisy was assessed by measuring the increased formation of prostaglandin E_2 in the pleural exudate. The levels of prostaglandin E_2 in carrageenan-treated rats was significantly increased and methylguanidine and aminoguanidine treatment significantly attenuated these levels (33.3% and 31.2%, respectively; Fig. 6B). Western blot analysis of lung homogenates obtained by carrageenan-treated rats also revealed an increase of cyclooxygenase-2 expression, which was significantly attenuated in lungs of methylguanidine-treated rats (42.6%; Fig. 8C,D).

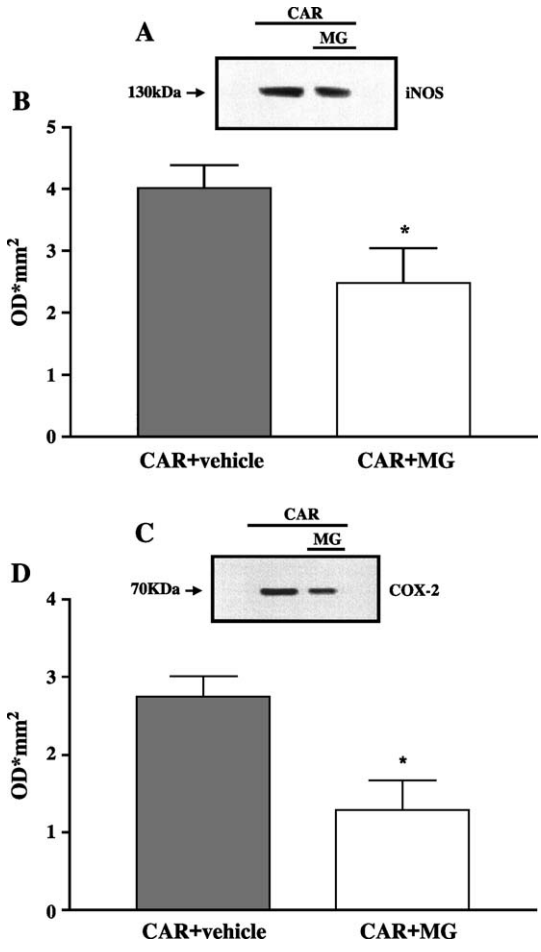

Fig. 8. A representative blot of lung iNOS (A) and cyclooxygenase-2 expression (C). Methylguanidine (30 mg/kg i.p.) significantly reduce iNOS (B) and cyclooxygenase-2 expression (D) in lung tissue. Data are means of mean ± S.E.M. from $n = 10$ rats for each group. *$P < 0.01$ vs. carrageenan.

4. Discussion

The inflammatory process is invariably characterised by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and by a release of chemicals from tissues and migrating cells (Vane and Botting, 1987; Tomlinson et al., 1994). Carrageenan-induced local inflammation is a commonly used to evaluate non-steroidal anti-inflammatory drugs (NSAID). Therefore, carrageenan-induced local inflammation (paw edema or pleurisy) is a useful model to asses the contribution of mediators involved in vascular changes associated with acute inflammation (Di Rosa and Willoughby, 1971). In particular, the initial phase of inflammation (edema, 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 and more recently has been attributed to the induction of cyclooxygenase-2 in the tissue (Di Rosa and 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., 1997b).

Furthermore, there is a large amount of evidence that the production of reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contribute to tissue damage (Hischi et al., 1989; 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 (Cuzzocrea et al., 1997b; Wei et al., 1995). Methylguanidine is a guanidine compound that has been found in meat extract, muscle autolyzates and in various tissue and biological fluids (Menichini et al., 1971). This compound, which accumulates in chronic renal failure, is a product of protein catabolism (Giovannetti et al., 1969) synthesized from creatinine by active oxygen generated not only by chemical reagents but also by isolated rat hepatocytes (Sakamoto et al., 1989). Previous studies reported that methylguanidine attenuates NO production by both constitutive and inducible isoforms of NOS (Sorrentino et al., 1997). In this study, treatment of rats with methylguanidine (30 mg/kg i.p.), as described above, did not modify rat blood pressure, indicating a lack effect on constitutive, endothelial NO production as previously demonstrated in lipopolysaccharide-induced shock (Autore et al., 1999).

Our results provides the evidence that methylguanidine attenuates: (i) the development of carrageenan-induced rat paw edema, (ii) the development of carrageenan-induced pleurisy, (iii) the infiltration of the lung with polymorpho-

nuclear leukocyte, (iv) the degree of lipid peroxidation in the lung, (v) the expression of iNOS and cyclooxygenase-2 and (vi) the degree of lung injury caused by injection of carrageenan. All of these findings support the view that methylguanidine attenuates the degree of acute inflammation in the rat. What, then, is the mechanism by which methylguanidine reduces acute inflammation?

A number of recent studies have demonstrated that the recruitment of cells into an area of inflammation may be mediated not only by C5a, leukotrienes, platelet-activating factor or bacterial-derived peptides, but also by a novel group of small proteins with relatively specific chemotactic activity for leukocyte subpopulations. We report in the present study 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 also demonstrated that treatment with methylguanidine reduces this inflammatory cells infiltration as assessed by the specific granulocyte enzyme myeloperoxydase 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 (Salvemini et al., 2001).

Furthermore, we found that the tissue damage induced by carrageenan in vehicle-treated rat was associated with high levels of tissue thiobarbituric acid-reactant malondialdehyde, which is considered a good indicator of lipid peroxidation (Ohkawa et al., 1979; Eiserich et al., 1996). An intense immunostaining of nitrotyrosine formation also suggested that a structural alteration of 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 (Eiserich et al., 1996; Cuzzocrea et al., 1999).

There is a large amount of evidence that the production of reactive oxygen species such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage (Salvemini et al., 1998; Holmdahl et al., 1985). 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 methylguanidine treatment prevent the induction of iNOS and the formation of peroxynitrite. Reactive oxygen species produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme poly(ADP-ribose) synthase 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 poly(ADP-ribose) synthase suicide hypothesis' (Szabò et al., 1997). There is recent evidence that the activation of poly(ADP-ribose) synthase may also play an important role in inflammation (Szabò et al., 1997, 1998; Wang et al., 1995) and we demonstrate here that methylguanidine treatment reduced the activation of poly(ADP-ribose) synthase during carrageenan-induced pleurisy in the lung. Thus, we propose that the anti-inflammatory effects of methylguanidine may be, at least in part, due to the prevention of the activation of poly(ADP-ribose) synthase.

In addition, there is evidence that the pro-inflammatory cytokines TNF-α help to propagate the extension of a local or systemic inflammatory process (Mannel and Echtenacher, 2000). We confirm here that the inflammatory process (carrageenan-induced pleurisy) leads to a substantial increase in the levels of TNF-α in the exudates which likely contribute in different capacities to the evolution of acute inflammation. Recently, studies have reported that treatment of monocytes and macrophages with high concentrations of methylguanidine reduced secretion of TNF-α and, in addition, in rats subjected to lipopolysaccharide-induced shock methylguanidine is able to reduce the production of TNF-α (Autore et al., 1999). Our data are in agreement with previously work since the pretreatment of rats with methylguanidine attenuated the production of TNF-α (Autore et al., 1999).

The data in this study support the notion that NO can increase cytokine expression. Recently, using iNOS-KO mice, it has been demonstrated that NO from iNOS may regulate cytokine formation and nuclear factor-κB (NF-κB) activation in hemorrhagic shock tissues (Hierholzer et al., 1998) as well as in ischemia and reperfusion injury (Cuzzocrea et al., 2002). Thus, it is likely that the NO-mediated signalling events that are initiated in early phases result in the rapid activation of downstream cascades. That iNOS regulation of inflammatory gene expression is perhaps a more generalized phenomenon is supported by a recent observation that the up-regulation of interferon and the response to IL-12 after *Leishmania major* infection is dependent on iNOS (Diefenbach et al., 1998). Our results, however, do not exclude the possibility that the observed differences in methylguanidine-treated rats are due to other mechanisms such us changes in organ perfusion and oxygen delivery resulting from reduced NO availability.

There is good evidence in this and in other models of inflammation that an enhanced formation of prostanoids following the induction of cyclooxygenase-2 contributes to the pathophysiology of inflammation (Salvemini et al., 1995; Sautebin et al., 1998) and also that selective inhibitors of cyclooxygenase-2 exert potent anti-inflammatory effects (Mitchell et al., 1993; Harada et al., 1996; Futaki et al., 1993). A cross talk between NOS and cyclooxygenase pathways has been suggested by many studies. Thus, NO has been reported to either negatively or positively modulate prostaglandins generation (Di Rosa et al., 1996; Salvemini et al., 1995).

In this study, we have shown that in carrageenan-induced pleurisy, almost in the absence of NO production, as in methylguanidine-treated rats, prostaglandin E_2 generation in the pleural exudate was significantly decreased compared to what was observed in carrageenan-treated rats. Thus, prostaglandin generation at the inflammation site seems to be, at least in part, under the control of NO. In fact in carrageenan-induced pleurisy NO is strongly generated by the iNOS, which is the predominant isoform (Tomlinson et al., 1994). Thus, our results suggest that the action of NO on prostaglandin biosynthesis seems to be dependent on iNOS induction. These results are in agreement with previous data, obtained in rat carrageenan pleurisy (Sautebin et al., 1998) and in other models of acute inflammation (Salvemini et al., 1994; Sautebin and Di Rosa, 1994, Sautebin et al., 1995a,b; Cuzzocrea et al., 1997a) showing that the modulation of NO pathway, either by inhibiting, with NOS inhibitors, or increasing, with L-arginine or NO donors, NO generation corresponds to a parallel modulation of prostaglandin biosynthesis.

Taken together, the findings confirm that induction of iNOS and subsequent production of NO contribute to acute inflammation. The pro-inflammatory properties of iNOS include the modulation of secretion of pro-inflammatory cytokines, production of pro-inflammatory prostaglandin, stimulation of neutrophil infiltration and related oxidative and nitrosative stress. This pro-inflammatory effect may be also attributed to a modulation of the cellular signalling mechanisms. In conclusion, this study provides the first evidence that methylguanidine causes a substantial reduction of acute inflammation in the rat.

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