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Effects of tempol, a membrane-permeable radical scavenger, in a rodent model of carrageenan-induced pleurisy

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Abstract

Carrageenan causes enhanced formation of reactive oxygen species, which contribute to the pathophysiology of inflammation. We have investigated the effects of tempol, a membrane-permeable radical scavenger, in rats subjected to carrageenan-induced pleurisy. Treatment of rats with tempol (10, 30, or 100 mg/kg 15 min prior to carrageenan) attenuated the pleural exudation and the migration of polymorphonuclear cells caused by carrageenan dose dependently. Tempol also attenuated the lung injury (histology) as well as the increase in the tissue levels of myeloperoxidase and malondialdehyde caused by carrageenan in the lung. However, tempol did not inhibit the activity of inducible nitric oxide synthase in the lungs. Immunohistochemical analysis for nitrotyrosine revealed positive staining in lungs from carrageenan-treated rats. Lung tissue sections from carrageenan-treated rats also showed positive staining for poly-(ADP-ribose) synthetase (PARS). The degree of staining for nitrotyrosine and PARS was markedly reduced in tissue sections obtained from carrageenan-treated rats, which had received tempol (100 mg/kg). Furthermore, treatment of rats with tempol significantly reduced (i) the formation of peroxynitrite, (ii) the DNA damage, (iii) the impairment in mitochondrial respiration, and (iv) the fall in the cellular level of NAD^+ observed in macrophages harvested from the pleural cavity of rats treated with carrageenan. Tempol also attenuated the cell injury caused by hydrogen peroxide (1 mM) in cultured human endothelial cells. This study provides the first evidence that tempol, a small molecule which permeates biological membranes and scavenges ROS, attenuates the degree of inflammation and tissue damage associated with carageenan-induced pleurisy in the rat. The mechanisms of the anti-inflammatory effect of tempol are discussed.

Keywords: Carrageenan; Inflammation; Nitric oxide (NO); Peroxynitrite; Superoxide dismutase; Tempol

1. Introduction

The role of reactive oxygen species in the pathophysiology of inflammation is well-established. In addition to ROS, overproduction of nitric oxide (NO) due to the expression of the inducible isoform of NO synthase (iNOS) also plays an important role in various models of inflammation (Moncada et al., 1991; Nathan, 1996; Cuzzocrea et al., 1998b). The local and systemic inflammatory response is also associated with the production of ROS such as superoxide anions, hydrogen peroxide and peroxynitrite (Youn et al., 1991; McCord, 1993; Cuzzocrea et al., 1998a). The biological activity and decomposition of peroxynitrite are very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, and other factors), and these factors influence its toxic potential (Beckman et

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al., 1990; Villa et al., 1994; Pryor and Squadrito, 1995). In a number of pathophysiological conditions associated with inflammation or oxidant stress, peroxynitrite has been proposed to mediate cell damage (Crow and Beckman, 1995; Miller et al., 1995; Zingarelli et al., 1997). Peroxynitrite is cytotoxic via a number of independent mechanisms including (i) the initiation of lipid peroxidation, (ii) the inactivation of a variety of enzymes (most notably, mitochondrial respiratory enzymes and membrane pumps) (Crow and Beckman, 1995) and (iii) depletion of glutathione (Phelps et al., 1995). Moreover, peroxynitrite can also cause DNA damage (Inoue and Kawanishi, 1995; Salgo et al., 1995) resulting in the activation of the nuclear enzyme poly-(ADP-ribose) synthetase (PARS), depletion of NAD and ATP and ultimately cell death (Zingarelli et al., 1996). The overproduction of ROS in inflammation leads to considerable oxidant stress, as indicated by lipid peroxidation, high blood levels of malondialdehyde and conjugated dienes as well as the consumption of the endogenous antioxidants vitamin C and vitamin E (Novelli, 1992; Cuzzocrea et al., 1997, 1999a).

Interventions, which reduce the generation or the effects of ROS, exert beneficial effects in a variety of models of inflammation including the carageenan-induced pleurisy model used here. These therapeutic interventions include melatonin (Cuzzocrea et al., 1997), a vitamin E-like antioxidant (Cuzzocrea et al., 1999a), a superoxide dismutase-mimetic (Cuzzocrea et al., 1999b), and a peroxynitrite decomposition catalyst (Salvemini et al., 1998). The therapeutic efficacy of SOD itself in animals with systemic inflammation, haemorrhage and shock is controversial. The following reasons may explain the lack of effect of SOD against the tissue injury associated with local or systemic inflammation. (1) SOD scavenges superoxide, but without efficient removal of the hydrogen peroxide that is produced, levels of hydroxyl radicals may increase (Goode and Webster, 1993). Indeed, SOD may function as a pro-oxidant by catalysing the conversion of hydrogen peroxide to hydroxyl radicals (Yim et al., 1990). (2) Neither SOD nor superoxide anions easily cross biological membranes. Thus, an increase in the amounts of extracellular SOD does not attenuate the effects of superoxide anions generated by intracellular sources (Fridovich, 1995). In contrast to SOD, spin trapping nitrones, such as phenyl *N-tert*-butyl nitrone (PBN), consistently improve the outcome in rat models of endotoxic (Mckechnie et al., 1986; Hamburger and McCay, 1989) and traumatic shock (Novelli, 1992; Novelli et al., 1986). Similarly, tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), a stable piperidine nitroxide (stable free radical) of low molecular weight, which permeates biological membranes and scavenges superoxide anions in vitro (Laight et al., 1997), also exerts beneficial effects in rats subjected to endotoxaemia (Leach et al., 1998). The effects of tempol (or other nitrones) in animal models of local inflammation have not yet been investigated.

Here we investigate the effects of tempol on the inflammatory response (pleurisy) caused by injection of carageenan in the rat. In addition, we have investigated the effects of tempol on the lung injury (histology), the formation of nitrotyrosine (immunohistochemistry) as well as the increases in iNOS and PARS activity caused by carageenan in the lung. In order to better understand the mechanisms of the observed anti-inflammatory effects of tempol, we have also investigated whether tempol attenuates the cell injury caused by oxidant stress (hydrogen peroxide) in human endothelial cells.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (300–350 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 purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986)

2.2. Experimental groups

In the treated group of animals, tempol was given as an intraperitoneal (i.p.) bolus 15 min before carrageenan (10, 30, or 100 mg kg^{-1}) (CAR + tempol group). In the vehicle-treated group of rats, vehicle (saline) was given instead of tempol (CAR group). In separate groups, surgery was performed identically in every aspect to the one in the CAR group, except that saline was injected instead of carrageenan (sham group; Sham). In an additional group of animals, sham surgery was combined with the administration of tempol (dose as above) (Sham + tempol).

2.3. Carrageenan-induced pleurisy

The rats were anaesthetised with isoflurane and a skin incision was made at the level of the left sixth intercostal space. The underlying muscle was dissected and saline (0.2 ml) or saline containing 1% λ-carrageenan (0.2 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 was rinsed with 2 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 was measured. Any exudate contaminated with blood was discarded. The amount of exudate was calculated by subtracting the volume injected (2 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 vital Trypan blue staining.

2.4. Cell culture

Resident pleural macrophages were collected 4 h after the carrageenan injection from rats treated or not with tempol (Cuzzocrea et al., 1998a). The cells (10^6 ml^{-1}), mainly macrophages (approximately 70%) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with L-glutamine (3.5 mM), penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}) and heparin sodium (10 U ml^{-1}) in 12-well plates for 2 h and were allowed to adhere at 37°C in a humidified 5% CO_2 incubator. Non-adherent cells were removed by rinsing the plates three times with 5% dextrose water. After removing non-adherent cells (approximately 10%), adherent macrophages were scraped for the measurement of DNA strand breaks and cellular NAD^+. Mitochondrial respiration and peroxynitrite formation were measured in the adherent cells in the subsequent 1-h period.

Human endothelial cells (EA.hy 926 cell line, passages 40 to 49) were seeded onto 96-well plates and cultured to confluence in DMEM containing glutamine (2 mM) and 10% foetal calf serum (FCS). Cell injury (i.e., reduction in mitochondrial respiration) was assessed with the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay (see below). Preliminary experiments were performed to evaluate the degree of cell injury induced by H_2O_2. In these experiments, the cells were exposed to various concentrations of H_2O_2 (0.03, 0.1, 0.3, 1, 3, 10 mM, $n = 4$, data not shown). In the subsequent intervention studies, the cells were challenged with a submaximal concentration of H_2O_2 (3 mM) for 4 h. The cells were pre-incubated (10 min prior to H_2O_2) in medium (1% FCS) in the absence or presence of increasing concentrations of tempol, deferoxamine mesylate or catalase. Controls (without exposure to H_2O_2) were included for all groups ($n = 4$).

2.5. Measurement of nitrite / nitrate

Nitrite + nitrate production, an indicator of NO synthesis, was measured in the supernatant samples as previously described (Cuzzocrea et al., 1998a). Briefly, the nitrate in the supernatant 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 nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylendiamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; vol. 1:1) to 100 μl samples. The optical density at 550 nm (OD_{550}) was measured using an ELISA microplate reader (SLT-Labinstruments Salzburg, Austria). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of DMEM.

2.6. Measurement of peroxynitrite-induced oxidation of dihydrorhodamine 123

The formation of peroxynitrite was measured by the peroxynitrite-dependent oxidation of dihydrorhodamine 123 to rhodamine 123, as previously described (Cuzzocrea et al., 1998a). Cells were rinsed with PBS and the medium was then replaced with PBS containing 5 μM dihydrorhodamine 123. After a 60-min incubation period at 37°C, the fluorescence of rhodamine 123 was measured using a fluorimeter at an excitation wavelength of 500 nm, emission wavelength of 536 nm (slit widths 2.5 and 3.0 nm, respectively). This method measures indirectly peroxynitrite production, as other oxidant species can also induce oxidation of dihydrorhodamine 123.

2.7. Measurement of mitochondrial respiration

Cell respiration was assessed by measuring the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Cuzzocrea et al., 1998a). Cells in 96-well plates were incubated at 37°C with MTT (0.2 mg ml^{-1}) for 1 h. Culture medium was removed by aspiration and the cells were solubilised in dimethyl sulfoxide (DMSO) (100 μl). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. As previously discussed (Darley-Usmar and Halliwell, 1996), the measurement of MTT reduction appears to involve mainly the mitochondrial complexes I and II, but may also involve NADH- and NADPH-dependent energetic processes that occur outside the mitochondrial inner membrane. Thus, this method cannot be used to separate the effect of free radicals, oxidants or other factors on the individual enzymes in the mitochondrial respiratory chain, but is useful to monitor changes in the general energetic status of the cells (Darley-Usmar and Halliwell, 1996).

2.8. Determination of DNA single-strand breaks

The formation of DNA strand breaks in double-stranded DNA was determined with alkaline unwinding methods as previously described (Schraufstatter et al., 1986; Cuzzocrea et al., 1998a). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (myoinositol 250 mM, NaH_2PO_3 10 mM, $MgCl_2$ 1 mM, pH 7.2). The cell lysate was then transferred into plastic tubes designated T (maximum fluorescence), P (fluorescence in sample used to estimate extent of DNA unwinding), or B (background fluorescence). To each tube, 0.2 ml of solution B (alkaline

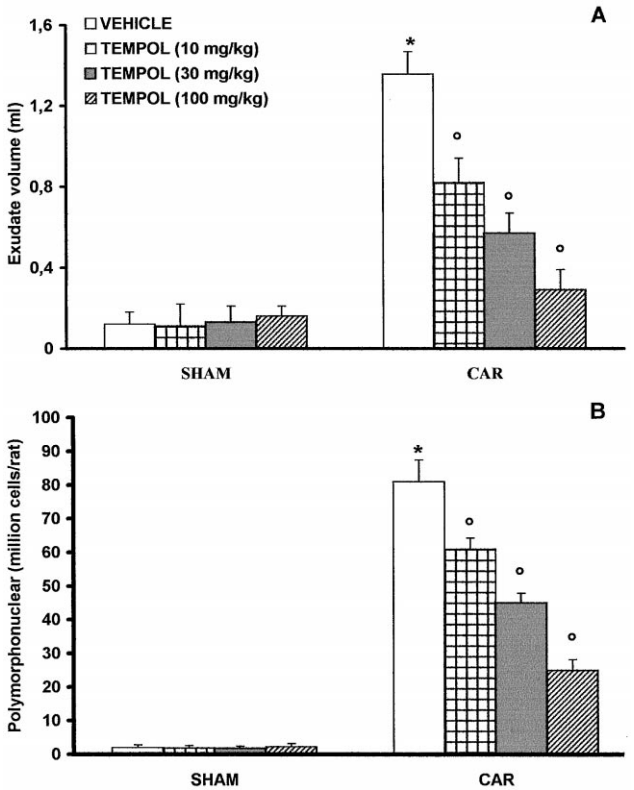

Fig. 1. Effect of tempol on carrageenan-induced inflammation. Volume exudate (A) and accumulation of polymorphonuclear cells (B) in pleural cavity at 4 h after carrageenan injection. Tempol (10, 30, or 100 mg/kg) significantly reduced pleural exudation and leukocyte infiltration in a dose-dependent fashion. Data are means ± S.E. means from 10 rats for each group. $^{*}P < 0.01$ vs. sham. $\circ P < 0.01$ vs. carrageenan.

lysis solution: NaOH 10 mM, urea 9 M, ethylenediaminetetraacetic acid 2.5 mM, sodium dodecyl sulphate 0.1%) was added and incubated at 4°C for 10 min to allow cell lysis and chromatin disruption. 0.1 ml each of solutions C (0.45 volume solution B in 0.2 N NaOH) and D (0.4 volume solution B in 0.2 N NaOH) were then added to the P and B tubes. 0.1 ml of solution E (neutralising solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D were added. From this point onwards, all incubations were carried out in the dark. A 30-min incubation period at 0°C was then allowed, during which the alkali diffused into the viscous lysate. As the neutralising solution, solution E, was added to the T tubes before addition of the alkaline solutions C and D, the DNA in the T tubes was never exposed to a denaturing pH. At the end of the 30-min incubation, the contents of the B tubes were sonicated for 30 s to ensure rapid denaturation of DNA in the alkaline solution. All tubes were then incubated at 15°C for 10 min. Denaturation was stopped by chilling to 0°C and by adding 0.4 ml of solution E to the P and B tubes. Solution F (1.5 ml) (ethidium bromide 6.7 μg/ml in 13.3 mM NaOH) was added to all the tubes and fluorescence (excitation: 520 nm, emission: 590 nm) was measured with a fluorimeter. Under the conditions used, in which ethidium bromide binds preferentially to double-stranded DNA, the percentage of double-stranded DNA (D) may be determined using the equation: $\%D = 100 \times [F(P) - F(B)]/[F(T) - F(B)]$; where F(P) is the fluorescence of the sample, F(B) the background fluorescence, i.e., fluorescence due to all cell components other than double-stranded DNA, and F(T) the maximum fluorescence.

2.9. Measurement of cellular NAD^+ levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 N $HClO_4$ scraped, neutralised with 3 M KOH, and centrifuged for 2 min at $10{,}000 \times g$. The supernatant was assayed for NAD^+ using a modification of the colorimetric method (Heller et al., 1995) in which NADH produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine

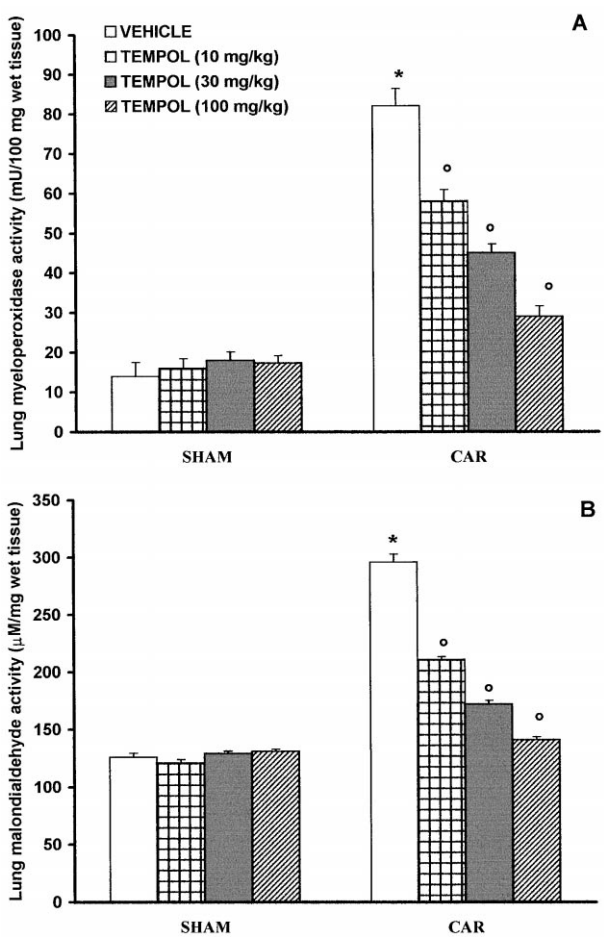

Fig. 2. Effect of tempol on myeloperoxidase activity and malondialdehyde levels in the lung. Myeloperoxidase (MPO) activity (A) and malondialdehyde (MDA) levels (B) in the lungs of carrageenan-treated rats killed at 4 h. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated rats in comparison to those of sham rats ($^{*}P < 0.01$). Tempol (10, 30, or 100 mg/kg) reduced the carrageenan-induced increase in MPO activity and MDA levels in a dose-dependent manner. Values are means ± S.E. means from 10 rats for each group. $^{*}P < 0.01$ vs. sham; $\circ P < 0.01$ vs. carrageenan.

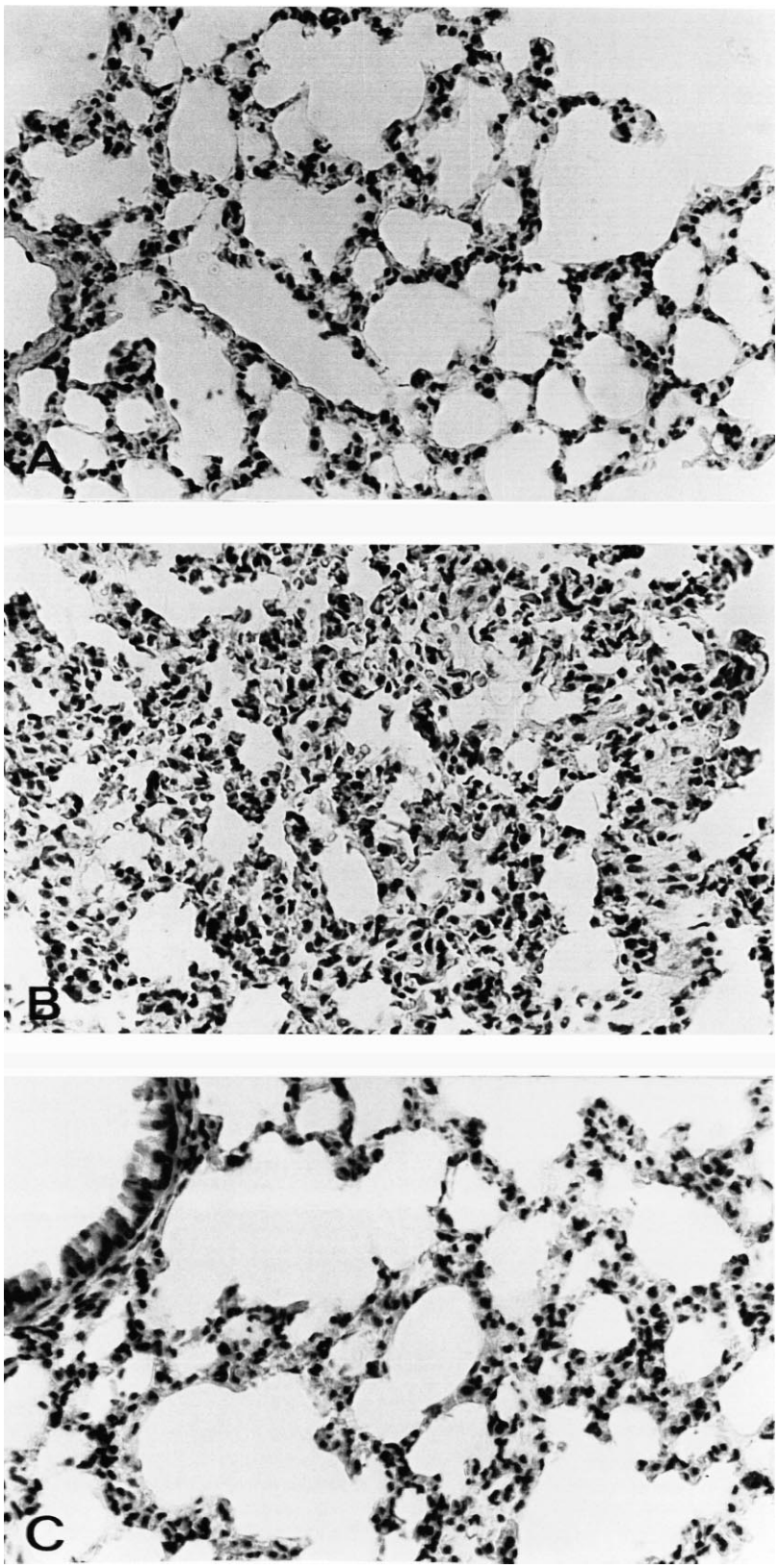

Fig. 3. Effect of tempol on lung injury. Representative lung sections from (A) sham-operated rats demonstrating a normal alveolar architecture. The lung section from a carrageenan-treated rat (B) shows interstitial haemorrhage and polymorphonuclear leukocyte accumulation. The lung section from a carrageenan-treated rat that had received tempol (100 mg/kg) (C) shows reduced interstitial haemorrhage and less cellular infiltration. Original magnification: ×150. Figure is representative of at least three experiments performed on different experimental days.

methasulfate. The rate of MTT reduction is proportional to the concentration of the co-enzyme. The reaction mixture contained 10 μl of a solution of 2.5 mg ml^{-1} MTT, 20 μl of a solution of 4 mg ml^{-1} phenazine methosulfate, 10 μl

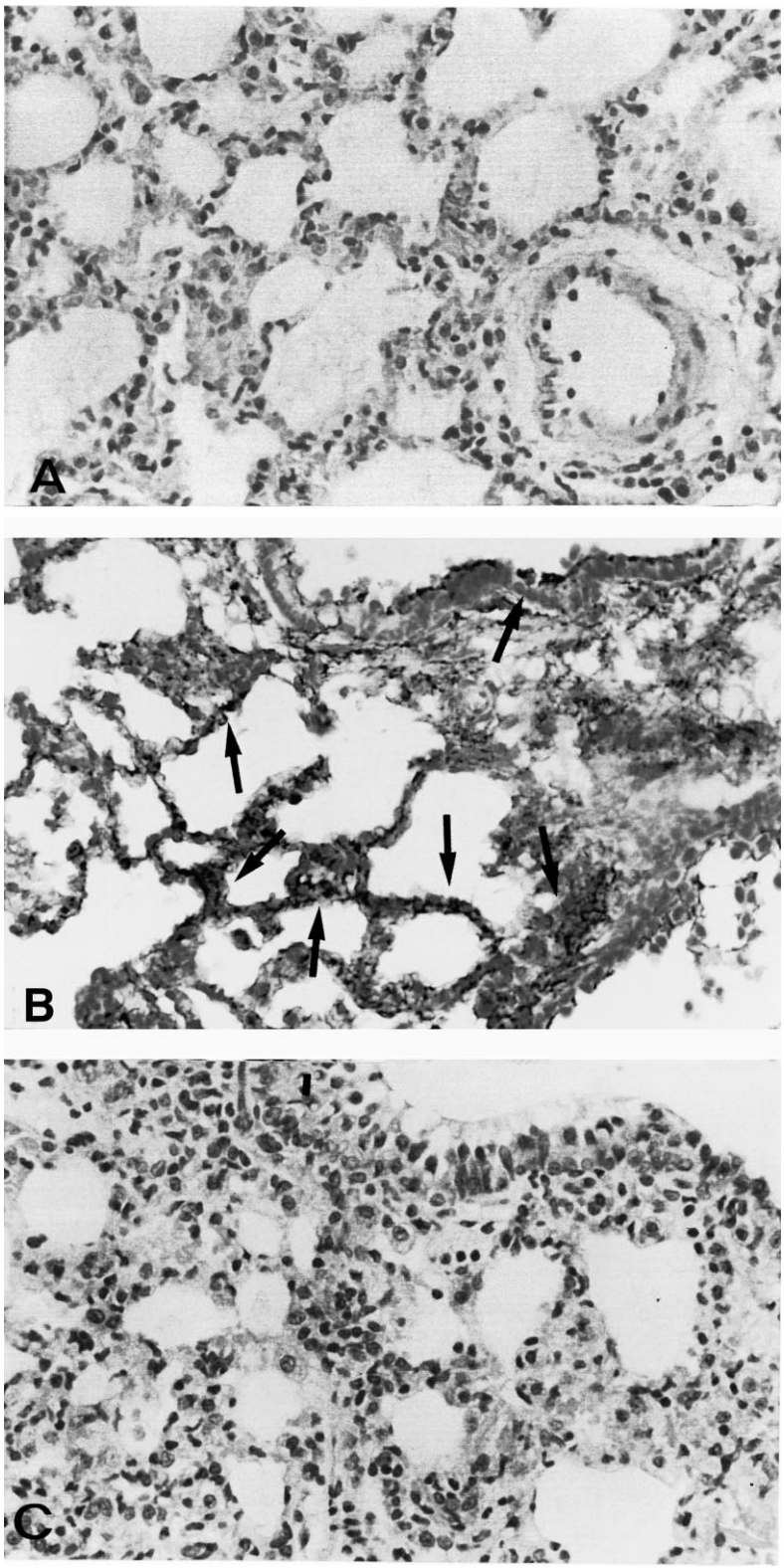

Fig. 4. Effect of tempol on nitrotyrosine formation. Immunohistochemical localisation of nitrotyrosine in the rat lung. Staining was absent in control tissue (A). At 4 h following carrageenan injection, positive staining for nitrotyrosine (typical areas are indicated by arrows) was observed (B). There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats pre-treated with tempol (100 mg/kg) (C). Original magnification: ×125. Figure is representative of at least three experiments performed on different days.

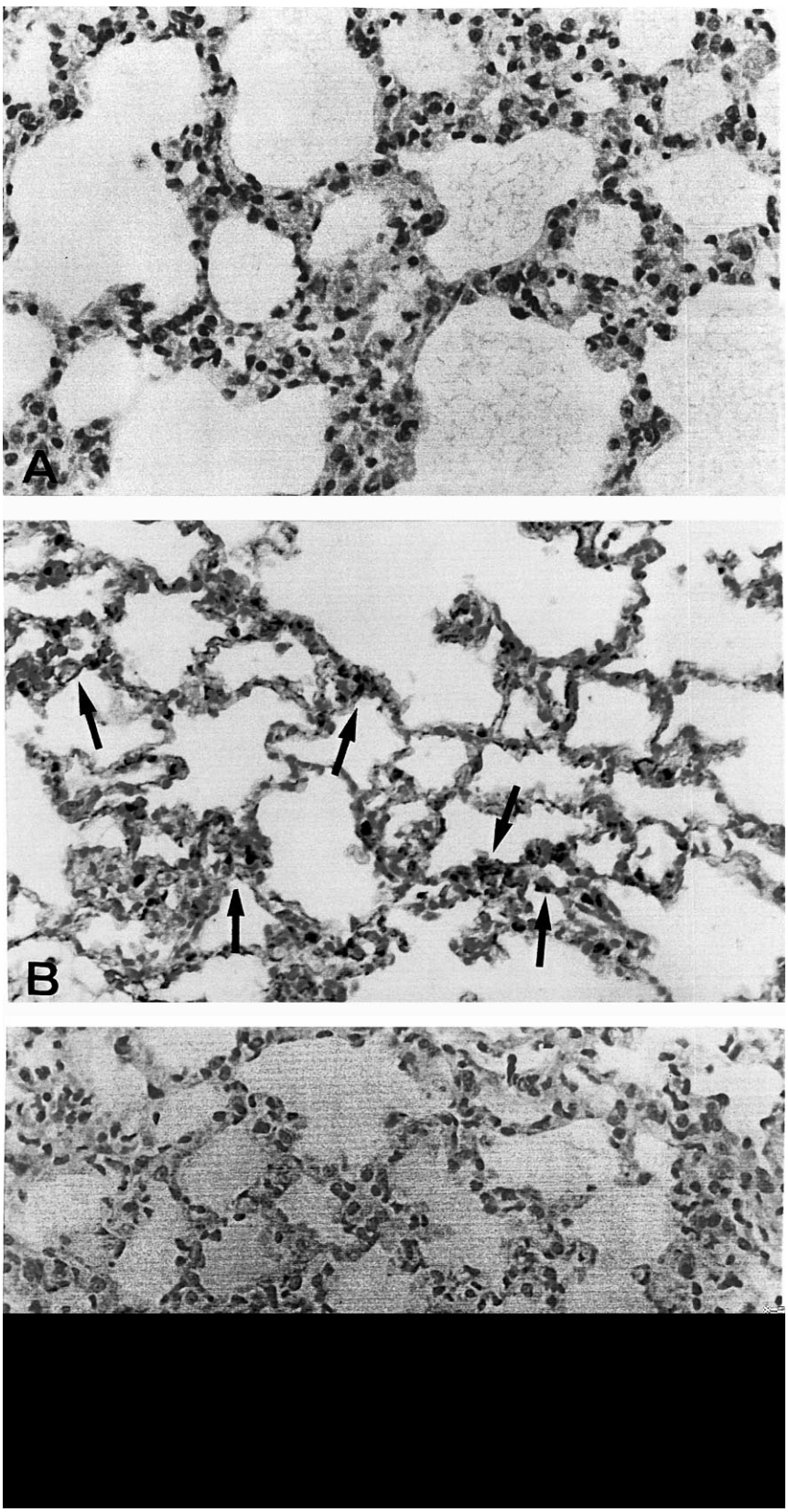

Fig. 5. Effect of tempol on PARS activity. Staining was absent in control tissue (A). At 4 h following carrageenan injection, PARS immunoreactivity was present in the lungs from carrageenan-treated rats (B). In the lungs of carrageenan-treated rats pre-treated with tempol (100 mg/kg) (C), no positive staining was found. Original magnification: ×150. Figure is representative of at least three experiments performed on different days.

of a solution of 0.6 mg ml^{-1} alcohol dehydrogenase (300 U mg^{-1}), and 190 μl of 0.065 M glycyl–glycine buffer, pH 7.4, that contained 0.1 M nicotinamide and 0.5 M ethanol. The mixture was warmed to 37°C for 10 min, and the reaction was started by the addiction of 20 μl of the sample. The rate of increase in absorbance was read immediately after the addition of NAD^+ samples and after 10- and 20-min incubation at 37°C against a blank at 560 nm in the ELISA microplate reader (SLT-Labinstruments Salzburg, Austria).

2.10. Histological examination

Lung biopsies were taken at 4 h after injection of carageenan. The biopsies were fixed for 1 week in buffered formaldehyde solution (10% in PBS) at room temperature, dehydrated with graded ethanol, and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness 7 μm) were deparaffinized with xylene, stained with trichromic Van Gieson and studied by light microscopy (Dialux 22 Leitz).

2.11. Immunohistochemical localisation of nitrotyrosine

Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or oxygen-derived free radicals, was determined by immunohistochemistry as previously described (Cuzzocrea et al., 1998b). At the end of the experiment, the relevant organs were fixed in 10% buffered formaldehyde 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 PBS for 20 min. Non-specific adsorption was minimised 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. The sections were then incubated overnight with 1:1000 dilution of primary anti-nitrotyrosine antibody 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.

2.12. Immunohistochemical localisation of PARS

At the specified time following the carrageenan injection, lung 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 PBS for 20 min. Non-specific adsorption was minimised 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 (DBA, Milan, Italy). The sections were then incubated overnight with 1:500 dilution of primary anti-poly (ADP-ribose) antibody (DBA) 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 (DBA).

2.13. Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte accumulation, was determined as previously described (Mullane et al., 1985). At the specified time following the intrapleural injection of carrageenan, lung tissues were obtained and weighed. Each piece of tissue was homogenised 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

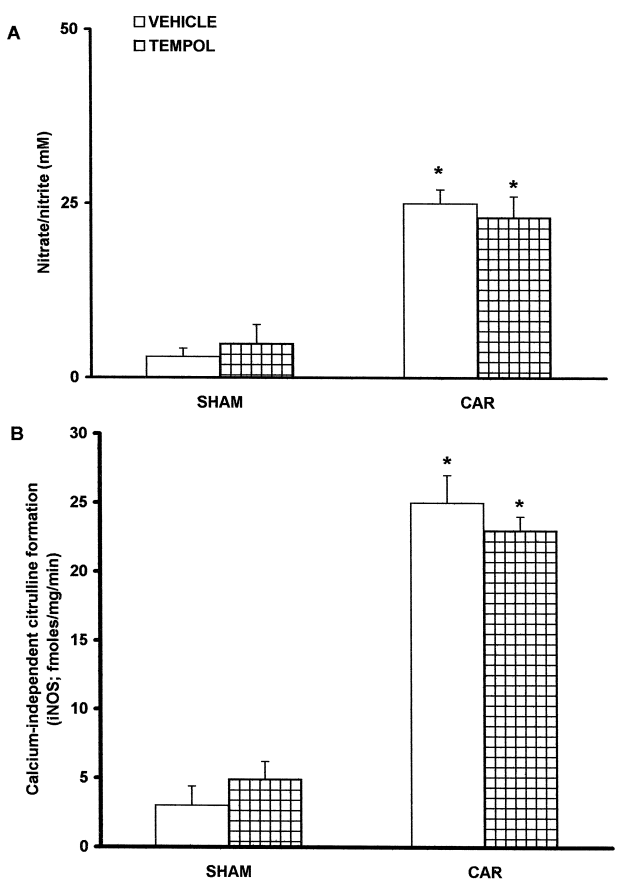

Fig. 6. Effect of tempol on NO formation by macrophage ex vivo. Nitrate/nitrite production (A), iNOS activity (B) in pleural macrophages harvested from control and carrageenan-treated rats. Production of NO was unaffected by pre-treatment of rats with tempol (100 mg/kg). * $P < 0.01$ vs. macrophages from control rats.

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 spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide min^{-1} at 37°C and was expressed in milliunits per gram weight of wet tissue.

2.14. Malondialdehyde measurement

The malondialdehyde level in the lung tissue samples was determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). Lung tissue, collected at the specified time, was homogenised in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% sodium dodecyl sulfate (SDS), 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid and 700 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 by spectrophotometry at 650 nm.

2.15. 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 the absence of tempol) served as an indicator of iNOS activity (Szabó et al., 1993). Cells were scraped into a homogenation buffer composed of 50 mM Tris–HCl, 0.1 mM EDTA and 1 mM phenylmethylsulphonyl fluoride (pH 7.4) and homogenised in the buffer on ice using a tissue homogenizer. Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described (Heller et al., 1995). 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 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 50 W (Na+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured with a Beckman scintillation counter.

2.16. Materials

Cell culture medium, heparin and FCS were obtained from Sigma (Milan, Italy). 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.

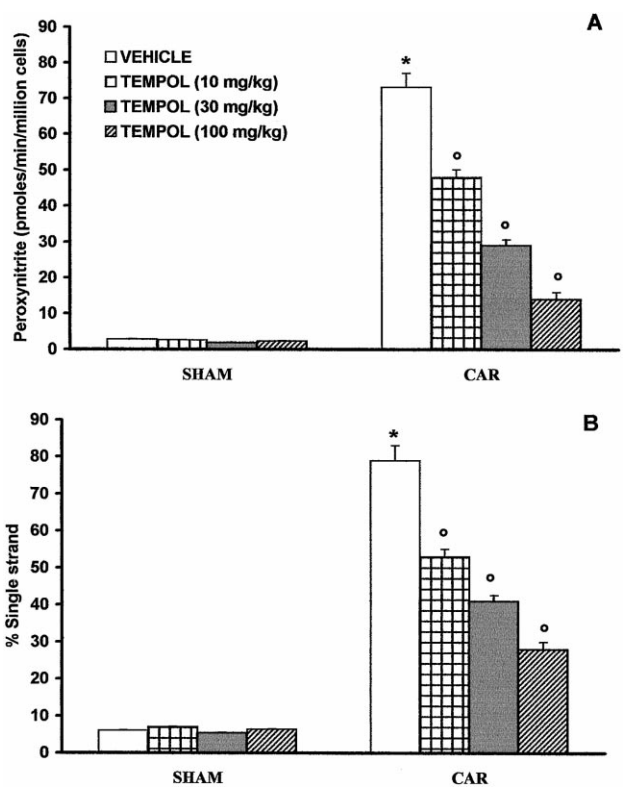

Fig. 7. Effect of tempol on peroxynitrite production and DNA damage. Peroxynitrite formation (A) and development of DNA single-strand breakage (B), in pleural macrophages harvested from control and carrageenan-treated rats. $^*P < 0.01$ vs. macrophages from control rats; $\circ P < 0.01$ represents a significant inhibitory effect of tempol.

2.17. Data analysis

All values in the figures and text are expressed as means ± S.E.M. of the mean of *n* observations. For the in vitro studies, the data represent the number of wells studied (six to nine wells from two to three independent experiments). 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 performed on different experimental days. The results were analysed by one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test for multiple comparisons. A *P*-value less than 0.05 was considered significant

3. Results

3.1. Effects of tempol in carrageenan-induced pleurisy

All rats, which were treated with carrageenan, developed acute pleurisy, characterised by the production of 1.41 ± 0.11 ml of turbid exudate (Fig. 1A). When compared to the number of cells collected from the pleural space of sham-operated rats ($2.2 \pm 0.7 \times 10^6$/rat, Fig. 1A), injection of carrageenan induced a significant increase in the number of PMNs ($83 \pm 5 \times 10^6$/rat, Fig. 1B). Pre-treatment of rats with tempol attenuated the volume of the pleural exudate as well as the number of PMNs within the exudate in a dose-related fashion (Fig. 1A,B).

In the lungs obtained from animals with carrageenan-induced pleurisy, a significant increase in iNOS activity was detected at 4 h after injection of carrageenan (213 ± 31 fmol/mg/min) (data not shown). Pre-treatment of rats with tempol did not affect this increase in iNOS activity (data not shown).

All rats which were treated with carrageenan exhibited a substantial increase in the activities of MPO and MDA in the lungs (Fig. 2A,B). Pre-treatment of rats with tempol attenuated the increase in MPO and MDA caused by carrageenan in the lung (Fig. 2A,B). In sham-operated rats, tempol had no effect on any of the parameters measured (Fig. 1A,B).

Histological examination of lung sections of rats treated with carrageenan showed oedema, tissue injury as well as infiltration of the tissue with PMNs, lymphocytes and plasma cells (Fig. 3B). Tempol treatment reduced both the lung injury and the infiltration of the tissue with white blood cells (Fig. 3C). Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan revealed positive staining for nitrotyrosine, which was primarily localised in alveolar macrophages and in airway epithelial cells (Fig. 4B). In contrast, no positive nitrotyrosine staining was found in the lungs of carrageenan-treated rats which had been pre-treated with tempol (Fig. 4C). Immunohistochemical analysis of lung sections obtained from rats treated with carrageenan also revealed positive staining for PARS (Fig. 5B). In contrast, no positive staining for PARS was found in the lungs of carrageenan-treated rats, which had been pre-treated with tempol (Fig. 5C). Please note that there was no staining for either nitrotyrosine or PARS in lungs obtained from sham-operated rats (Figs. 4A, 5A).

3.2. Effects of tempol on the increase in peroxynitrite formation, iNOS induction, DNA damage and injury of macrophages obtained from the pleural cavity of carrageenan-treated rats

When compared to the supernatant of macrophages collected from the pleural cavity of sham-operated animals, the supernatant of macrophages from carrageenan-treated rats showed a significant increase in the concentration of nitrite + nitrate (Fig. 6A). This was associated with a significant increase in iNOS activity in these cells (Fig. 6B). Pre-treatment of rats (in vivo) with tempol did not affect the increase in nitrite + nitrate and the induction of iNOS activity (Fig. 6).

When compared to the supernatant of macrophages collected from the pleural cavity of sham-operated ani-

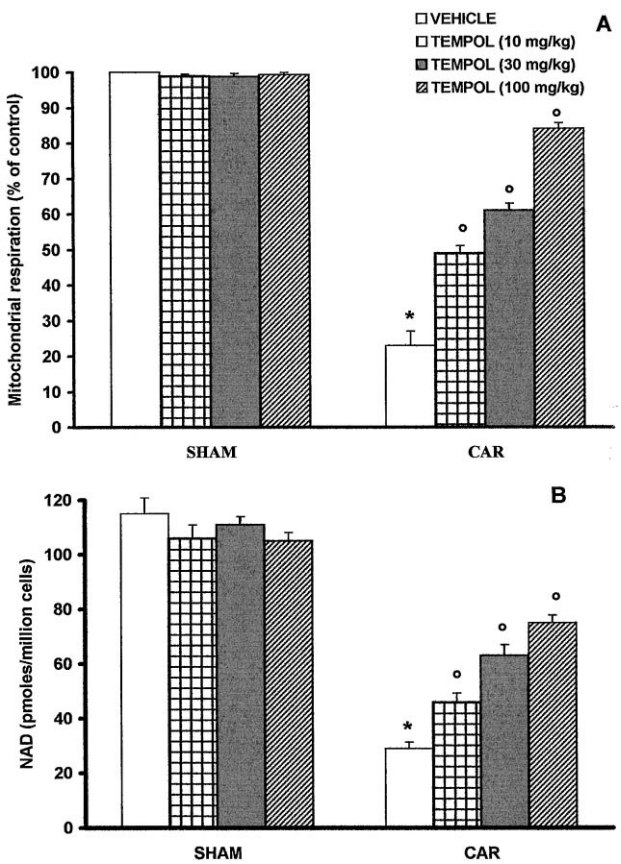

Fig. 8. Effect of tempol on cellular dysfunction. Reduction of mitochondrial respiration (A) and cellular levels of NAD^+ (B) in macrophages from control and carrageenan-treated rats. $^*P < 0.01$ vs. macrophages from control rats; $\circ P < 0.01$ represents protective effects of tempol.

mals, the supernatant of macrophages obtained from carrageenan-treated rats showed a significant increase in the concentration of peroxynitrite (Fig. 7A). This was associated with a significant increase in the occurrence of single-strand breaks in the DNA (Fig. 7B), a reduction in mitochondrial respiration (Fig. 8A), as well as a significant fall in the intracellular levels of NAD (Fig. 8B) in these cells. Pre-treatment of rats (in vivo) with tempol attenuated the formation of peroxynitrite as well as the associated DNA damage (Fig. 7), impairment of mitochondrial respiration (Fig. 8A) as well as the fall in NAD levels (Fig. 8B) in a dose-dependent manner.

3.3. Effects of tempol on the impairment in mitochondrial respiration caused by hydrogen peroxide in human endothelial cells

Exposure of endothelial cells to hydrogen peroxide (1 mM) for 4 h caused a substantial impairment in mitochondrial respiration (Fig. 9A). The reduction in mitochondrial respiration caused by hydrogen peroxide in these cells was attenuated (to a similar degree) by tempol (Fig. 9A) and desferoxamine (Fig. 9B), and abolished by catalase (Fig. 9C).

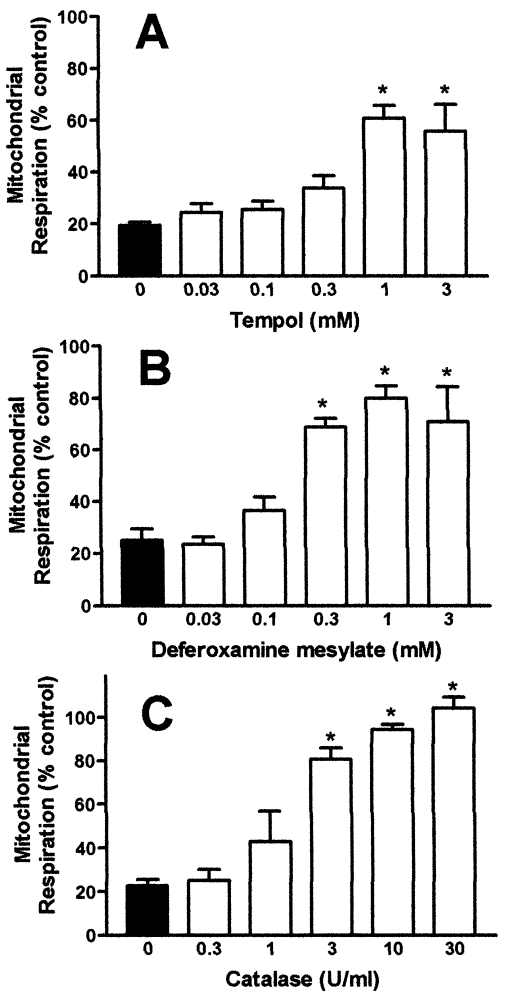

Fig. 9. Effect of tempol (A) on the impairment in mitochondrial respiration caused by hydrogen peroxide: Comparison with deferroxamine (B) and catelase (C). Cultured human endothelial cells were pre-treated with the relevant drugs and were exposed (15 min later) to hydrogen peroxide (1 mM for 4 h).

4. Discussion

The inflammatory process is invariably characterised by production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and interleukin 1 and by a release of chemicals from tissues and migrating cells (Vane and Botting, 1987; Tomlinson et al., 1994). Furthermore, there is much evidence that the production of ROS such as hydrogen peroxide, superoxide and hydroxyl radicals at the site of inflammation contributes to tissue damage (Oh-Ishi et al., 1989; Dawson et al., 1991; Peskar et al., 1991; Da Motta et al., 1994; Salvemini et al., 1996; Cuzzocrea et al., 1997, 1998a). 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 (Tracey et al., 1995; Wei et al., 1995; Salvemini et al., 1996; Cuzzocrea et al., 1997, 1998a). In addition to NO, peroxynitrite is also generated in carrageenan-induced inflammation (Salvemini et al., 1996; Cuzzocrea et al., 1997, 1998a,b). The biological activity and decomposition of peroxynitrite is very much dependent on the cellular or chemical environment (presence of proteins, thiols, glucose, the ratio of NO and superoxide, carbon dioxide levels and other factors), and these factors influence its toxic potential (Beckman et al., 1990; Rubbo et al., 1994; Villa et al., 1994; Pryor and Squadrito, 1995). We now demonstrate that tempol reduces (i) the development of carrageenan-induced pleurisy, (ii) the infiltration of the lung with PMNs (histology and MPO activity), (iii) the degree of lipid peroxidation in the lung, and (iv) the degree of lung injury (histology) in rats treated with carrageenan. All these findings support the view that tempol attenuates the degree of inflammation and lung injury caused by carrageenan in the rat. What, then, is the mechanism by which tempol protects the lung against this inflammatory injury? Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) is a stable piperidine nitroxide (stable free radical) of low molecular weight, which permeates biological membranes and scavenges superoxide anions in vitro (Laight et al., 1997). In addition, tempol inhibits the catalytic action of transition metal irons and hence, attenuates the formation of hydroxyl radicals (Monti et al., 1996). Similarly, tempol protects cultured rabbit epithelial cells against the injury caused by hydrogen peroxide, which is also mediated by hydroxyl radicals (Reddan et al., 1992). Thus, tempol scavenges intracellular superoxide anions and prevents the formation of hydroxyl radicals. Unlike recombinant SOD,

which is not able to cross biological membranes, tempol crosses biological membranes and functions as an intracellular scavenger of superoxide anions and other radicals. We therefore propose that the beneficial effects of tempol on pleurisy are also due to the ability of this stable, free nitroxide radical to function as an intracellular scavenger of superoxide anions and other free radical species. Indeed, we demonstrate here that tempol abolishes the injury caused by hydrogen peroxide in human endothelial cells. This injury is secondary to the formation of hydroxyl radicals, as it is also prevented by deferroxamine, an agent which attenuates the formation of hydroxyl radicals from hydrogen peroxide. In addition, we demonstrate that tempol attenuates the nitrosylation of proteins in the lung of rats treated with carrageenan. 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 (Beckman, 1996). There is, however, recent evidence that certain other reactions can also induce tyrosine nitration; e.g., the reaction of nitrite with hypochlorous acid and the reaction of myeloperoxidase with hydrogen peroxide can lead to the formation of nitrotyrosine (Eiserich et al., 1998). Increased nitrotyrosine staining is, therefore, considered an indication of ''increased nitrosative stress'' rather than a specific marker of the generation of peroxynitrite.

ROS and peroxynitrite produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage. 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 co-factor 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* (see Section 1). There is recent evidence that the activation of PARS may also play an important role in inflammation (Szabó et al., 1997, 1998; Cuzzocrea et al., 1998a,c). We demonstrate here that tempol attenuates the increase in PARS activity caused by carrageenan in the lung. Similarly, tempol attenuates the formation of peroxynitrite by macrophages (ex vivo) obtained from rats injected with tempol. In these macrophages, tempol also attenuated the fall in NAD associated with the enhanced formation of peroxynitrite. Thus, we propose that the anti-inflammatory effects of tempol reported here are — at least in part — due to the prevention of the activation of PARS.

In conclusion, this study demonstrated that the stable nitroxide radical, tempol, attenuates the pleurisy caused by carrageenan administration in the rat. We speculate that the observed anti-inflammatory effects of tempol may be dependent on a combination of the following pharmacological properties of this agent: (1) tempol scavenges and inactivates superoxide anions, which would prevent the formation of peroxynitrite. This, in turn, prevents the activation of PARS and the associated tissue injury. (2) In addition to superoxide anions, tempol also scavenges other ROS, including hydroxyl radicals. It is not clear whether tempol is able to scavenge peroxynitrite. (3) In addition, tempol reduces the recruitment of PMNs into the inflammatory site. This effect of tempol is very likely secondary to the prevention by tempol of endothelial oxidant injury and, hence, preservation of endothelial barrier function. These results support the view that the overproduction of reactive oxygen or nitrogen free radicals contributes to acute inflammation. Finally, we propose that small molecules, such as tempol, which permeate biological membranes and function as intracellular radical scavengers, may be useful in the therapy of conditions associated with local or systemic inflammation.

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