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Beneficial effects of raxofelast (IRFI 016), a new hydrophilic vitamin E-like antioxidant, in carrageenan-induced pleurisy

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1 Peroxynitrite is a strong oxidant that results from reaction between NO and superoxide. It has been recently proposed that peroxynitrite plays a pathogenetic role in inflammatory processes. Here we have investigated the therapeutic efficacy of raxofelast, a new hydrophilic vitamin E-like antioxidant agent, in rats subjected to carrageenan-induced pleurisy.

2 *In vivo* treatment with raxofelast (5, 10, 20 mg kg^{-1} intraperitoneally 5 min before carrageenan) prevented in a dose dependent manner carrageenan-induced pleural exudation and polymorphonuclear migration in rats subjected to carrageenan-induced pleurisy. Lung myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels, as well as histological organ injury were significantly reduced by raxofelast.

3 Immunohistochemical analysis for nitrotyrosine, a footprint of peroxynitrite, revealed a positive staining in lungs from carrageenan-treated rats. No positive nitrotyrosine staining was found in the lungs of the carrageenan-treated rats, which received raxofelast (20 mg kg^{-1}) treatment.

4 Furthermore, *in vivo* raxofelast (5, 10, 20 mg kg^{-1}) treatment significantly reduced peroxynitrite formation as measured by the oxidation of the fluorescent dihydrorhodamine 123, prevented the appearance of DNA damage, the decrease in mitochondrial respiration and partially restored the cellular level of NAD^+ in *ex vivo* macrophages harvested from the pleural cavity of rats subjected to carrageenan-induced pleurisy.

5 In conclusion, our study demonstrates that raxofelast, a new hydrophilic vitamin E-like antioxidant agent, exerts multiple protective effects in carrageenan-induced acute inflammation.

Keywords: Raxofelast; peroxynitrite; carrageenan; free radicals; inflammation

Abbreviations: ecNOS, constitutive endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; MPO, myeloperoxidase; NO, nitric oxide; NOS, nitric oxide synthase; PARS, poly (ADP-ribose) synthetase; PMN, polymorphonuclear cells

Introduction

The role of oxyradical formation in various forms of inflammation is well established. Recent data demonstrate that the expression of the inducible isoform of nitric oxide (NO) synthase also plays important pathogenetic roles in various models of inflammation (Moncada *et al*., 1991; Nathan 1992; Cuzzocrea *et al*., 1998a). The systemic inflammatory response is also associated with the production of oxygen-derived free radicals (Youn *et al*., 1991; McCord, 1993), and there is now substantial evidence that much of the cytotoxicity is due to a concerted action of oxygen- and nitrogen-derived free radicals and oxidants. Peroxynitrite, a cytotoxic oxidant species formed from the reaction of NO and superoxide (Beckman *et al*., 1990) may mediate part of the oxidative injury associated with simultaneous production of NO and oxyradicals. 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; Villa *et al*., 1994; Rubbo *et al*., 1994; Pryor & Squadrito, 1995). Peroxynitrite formation has been demonstrated in various inflammatory disorders (Halliwell, 1995; Salvemini *et al*., 1996a,b; Cuzzocrea *et al*., 1997b,c; 1998a) and in circulatory shock (Wiseman *et al*., 1994; Szabò, 1996).

Using the experimental model described here, previous work has demonstrated the anti-inflammatory potential of various therapeutic approaches aimed at the inhibition of NO synthesis and peroxynitrite formation (Tracey *et al*., 1995; Cuzzocrea *et al*., 1997c; 1998a). In our studies we utilized raxofelast (IRFI 016; 5-acetyloxy-2,3-dihydro-4,6,7-trimethyl-2-benzofuranacetic acid; Figure 1) a new hydrophilic vitamin E-like antioxidant agent (Campo *et al*., 1997). It was selected from a series of new compounds (Ceccarelli *et al*., 1993) designed with the aim of maximizing the antioxidant potency of phenols chemically related to α-tocopherol (vitamin E). The antioxidant activity of raxofelast has been convincingly demonstrated in several *in vitro* studies (Mattioli *et al*., 1991) and in various models of ischaemia-reperfusion injury (Campo *et al*., 1992; 1994). The purpose of the present study was to investigate the protective effect of raxofelast against the cellular energetic failure and the development of inflammation in rats treated with carrageenan.

Methods

Experimental groups

In the treated group of animals, raxofelast, was given intraperitoneally (i.p.) 5 min before carrageenan (5, 10, 20 mg kg^{-1}) (carrageenan + raxofelast group). In a vehicle-treated group of rats, vehicle (saline) was given instead of raxofelast (carageenan group). In separate groups of rats, surgery was performed in its every aspect identical to the one in

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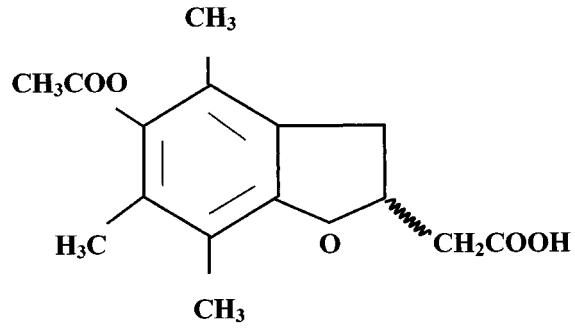

2,3-dihydro-5-acetyloxy-4,6,7-trimethyl-2-benzofuranacetic acid (IRFI-016)

Figure 1 Chemical structure of IRFI 016 (raxofelast).

the carrageenan group, except that carrageenan was not injected (control group). In an additional group of animals, control surgery was combined with the administration of raxofelast (dose as above) (control + raxofelast).

Carrageenan-induced pleurisy

Rats were lightly anaesthetized under isoflurane 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% λ-carrageenan were 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 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^{-1}) and indomethacin (10 μg ml^{-1}). The exudate and washing 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. Leucocytes in the exudate were suspended in phosphate buffer saline and counted with an optical microscope using a Burker's chamber after vital Trypan Blue stain.

Cell culture

Resident pleural cells macrophages were collected 4 h after the carrageenan injection from rats treated with or without raxofelast (Cuzzocrea *et al*., 1998b). The cells (1×10^6 ml^{-1}), being mainly macrophages (approximately 70%) were cultured in DMEM medium, supplemented with M-glutamine (3.5 mM), penicillin (50 u ml^{-1}, streptomycin (50 μg ml^{-1}) and heparin sodium (10 u ml^{-1}) in 12-well 2 h and allowing cells to adhere at 37°C in a humidified 5% CO_2 incubator. Nonadherent cells were removed by rinsing the plates three times with 5% dextrose water. After removing nonadherent cells (approximately 10%), adherent macrophages were scraped from 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.

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*., 1998b). Cells were rinsed with phosphate-buffered saline and then medium was replaced with phosphate-buffered saline containing 5 μM dihydrorhodamine 123. After a 60 min incubation 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). Thus this method is an indirect measurement of peroxynitrite production because also other oxidant species can induce oxidation of dihydrorhodamine 123.

Measurement of mitochondrial respiration

Cell respiration was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] to formazan (Zingarelli *et al*., 1996). 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 solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of OD_{550}. As previously discussed (Darley-Usmar & Halliwell, 1996), the measurement of reduction of MTT appears to be mainly by the mitochondrial complexes I and II, it also may 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 & Halliwell, 1996).

Determination of DNA single-strand breaks

The formation of DNA strand breaks in double-stranded DNA was determined by the alkaline unwinding methods as previously described (Zingarelli *et al*., 1996; Schraufstatter *et al*., 1986). Cells in 12-well plates were scraped into 0.2 ml of solution A buffer (myoinositol 250 mM, NaH_2PO_3 10 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 lysis solution: NaOH 10 mM, urea 9 M, ethylenediamineteraacetic 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 (neutralizing solution: glucose 1 M, mercaptoethanol 14 mM) was added to the T tubes before solutions C and D were added. From this point 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. Since the neutralizing 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 adding 0.4 ml of solution E to the P and B tubes. 1.5 ml of solution F (ethidium bromide 6.7 μg ml^{-1} in 13.3 mM NaOH) was added to all the tubes and fluorescence (excitation: 520 nm, emission: 590 nm) was measured by 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.

Measurement of cellular NAD$^+$ levels

Cells in 12-well plates were extracted in 0.25 ml of 0.5 N $HC10_4$ scraped, neutralized 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 methosulphate. 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 methosulphate, 10 μl 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 addition 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 blank at 560 nm in the ELISA microplate reader (SLT-Labinstruments Salzburg, Austria).

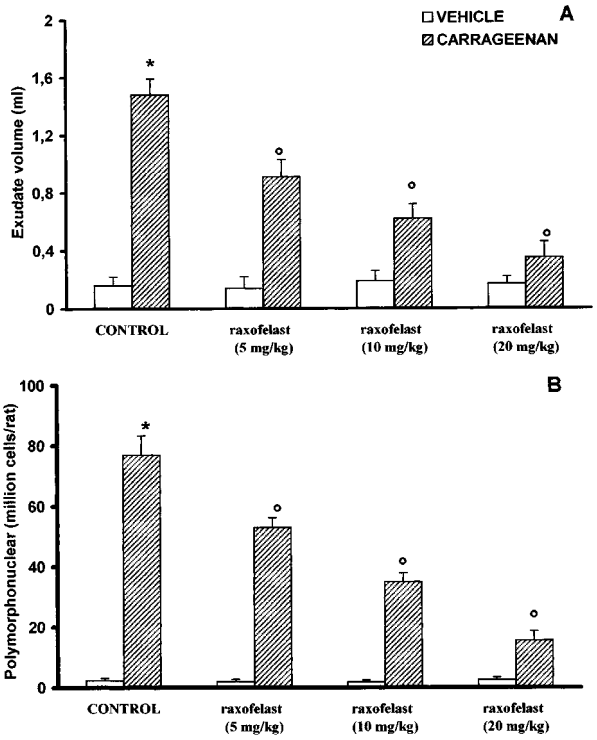

Figure 2 Volume exudate (A) and polymorphonuclear accumulation (B) in pleural cavity at 4 h after carrageenan injection. Raxofelast (5, 10, 20 mg kg^{-1}) treatment significantly reduced in a dose dependent manner pleural exudation and leukocyte migration. Data are means ± s.e.mean of ten rats for each group. *$P<0.01$ versus control; °$P<0.01$ versus carrageenan.

Light microscopy

Lung biopsies were taken 4 h after the induction of pleurisy by carrageenan injection. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ, U.S.A.). Section (thickness 7 μm) were deparaffinized with xylene, stained with trichromic Van Gieson and observed in Dialux 22 Leitz microscope.

Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, a specific 'footprint' of peroxynitrite formation, was detected as previously described (Cuzzocrea *et al*., 1997b) 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. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate

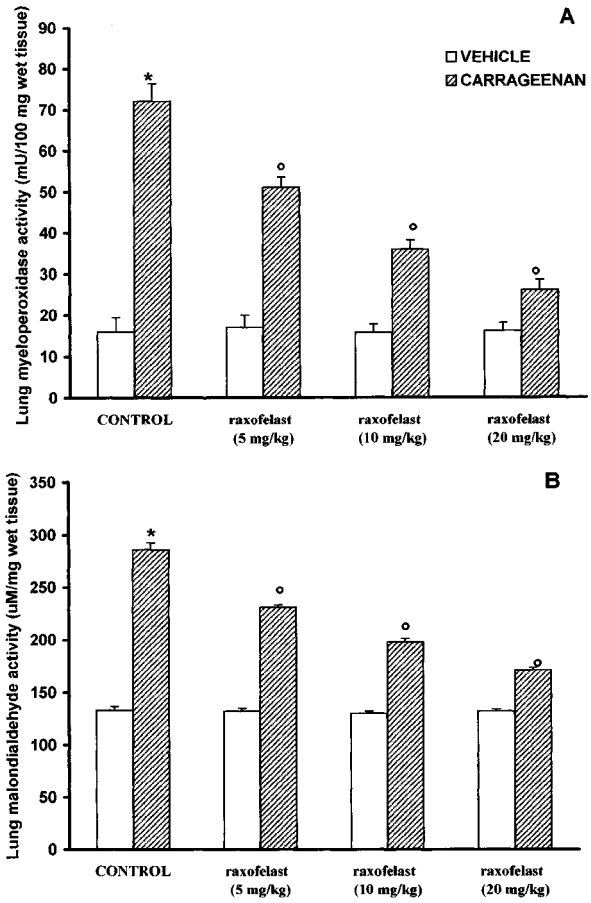

Figure 3 Myeloperoxidase (MPO) (A) and malondialdehyde (MDA) (B) in the lungs of carrageenan-treated rats. MPO activity and MDA levels were significantly increased in the lungs of the carrageenan-treated rats in comparison to control rats raxofelast (5, 10, 20 mg kg^{-1}) significantly reduced the carrageenan-induced increase in MPO activity and MDA levels. Values are means ± s.e.mean of ten rats for each group. *$P<0.01$ versus control; °$P<0.01$ versus carrageenan.

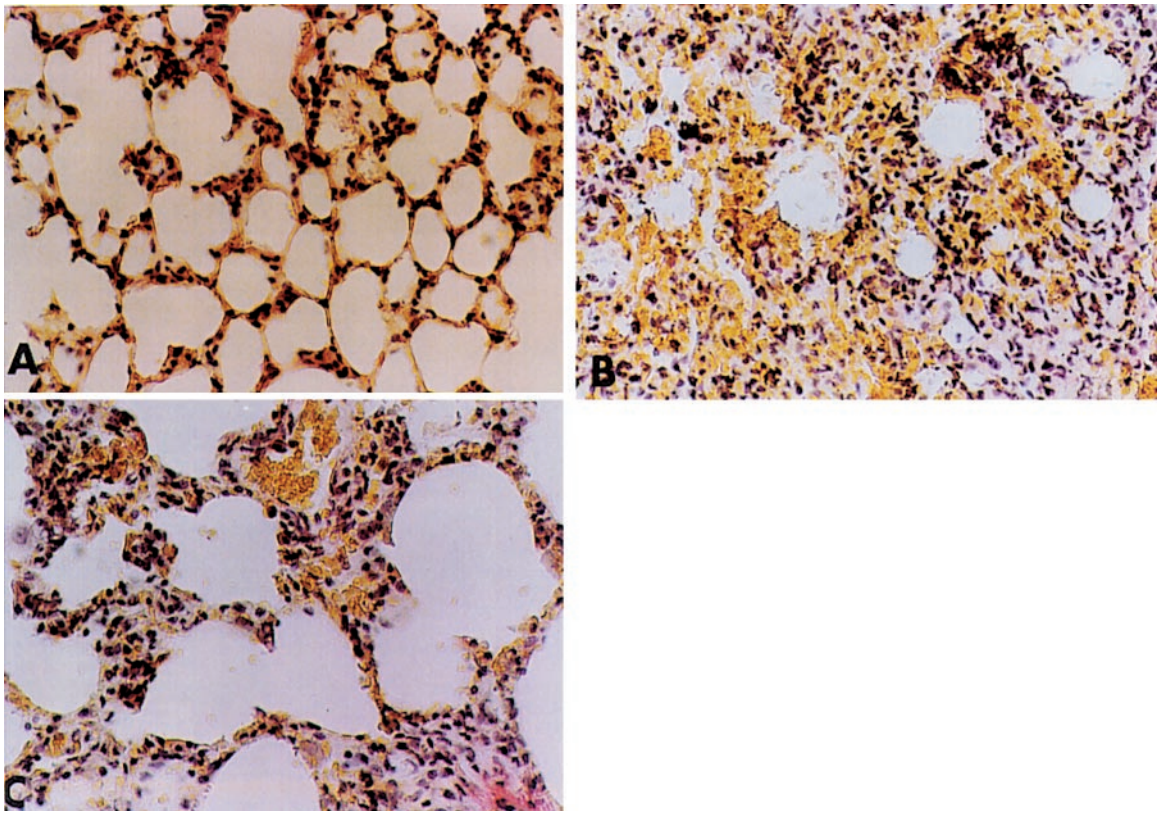

Figure 4 Representative lung sections from (A) control rats demonstrating the normal alveolar architecture. Lung sections from a carrageenan-treated rat (B) demonstrate inflammatory infiltration by neutrophil, lymphocytes and plasma. Lung sections from a carrageenan-treated rat that received raxofelast (20 mg kg^{-1}) (C) demonstrate reduced inflammatory infiltration. Original magnification: $\times 62.5$. Figure is representative of at least three experiments performed on different experimental days.

buffered saline 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 : 1000 dilution of primary anti-nitrotyrosine antibody (DBA, Milan, Italy) 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 (DBA, Milan, Italy).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an index of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Mullane *et al*., 1988). At the specified time following the intrapleural injection of carrageenan lung tissues, were obtained and weighed. Each piece of tissue was homogenized in a solution containing 0.5% hexa-decyltrimethyl-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 spectrophotometrically at 650 nm. Myeloperoxidase 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.

Malondialdehyde (MDA) measurement

Malondialdehyde (MDA) levels in the lung tissue were determined as an index of lipid peroxidation, as described by Okhawa *et al*. (1979). Lung tissue, collected at the specified time, were homogenized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% 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.

Materials

Raxofelast was supplied by Biomedica Foscama, Ferentino (FR), Italy. Cell culture medium, heparin and foetal calf serum were obtained from Sigma (Milan, Italy). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, primary anti-nitrotyrosine antibody and avidin-biotin peroxidase complex were obtained from 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 $\pm$ standard error (s.e.m.) of the mean of n observations. For the *in vitro* studies, data represent the number of wells studied (6–9 wells from 2–3 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 ANOVA followed by a *post-hoc*

Bonferroni test. A P-value less than 0.05 was considered significant.

Results

Effects of raxofelast in carrageenan-induced pleurisy

All carrageenan-injected rats developed an acute pleurisy, producing 1.8 ± 0.15 ml of turbid exudate (Figure 2A). Trypan blue stain revealed $77 \pm 6.4 \times 10^6$ rat^{-1} PMNs in comparison to control rat ($2.4 \pm 0.8 \times 10^6$ rat^{-1}) (Figure 2B). Control animals demonstrated no abnormalities in the pleural cavity or fluid. The degree of peritoneal exudation and polymorphonuclear migration were significantly reduced in rats with raxofelast (Figure 2A,B). Raxofelast treatment did not cause significant changes in these parameters in control rats (Figure 2A,B).

Lungs obtained from carrageenan-treated rats were examined for the measurement of MPO activity, the latter being indicative of neutrophil infiltration, and for MDA levels, in order to estimate lipid peroxidation. As shown in Figure 3A, B, MPO activity and MDA levels (7.2 ± 6.4 mu 100 mg^{-1} wet tissue and 286 ± 4.4 μM mg^{-1} wet tissue, respectively) were significantly ($P < 0.01$) increased in the lung from carrageenan-treated rats when compared to control rats (16 ± 3.5 mu 100 mg^{-1} wet tissue; 133 ± 6.8 μM mg^{-1} wet tissue respectively.) MPO activity and MDA levels were significantly ($P < 0.01$) reduced in a dose dependent manner by raxofelast treatment (Figure 3A,B).

Histological examination of lung section showed inflammatory infiltration by neutrophil, lymphocytes and plasma cells extravasation (Figure 4B). Raxofelast treatment reduced histological organ injury (Figure 4C).

Lung sections were also analysed for the presence of nitrotyrosine, a footprint of peroxynitrite. Immunohistochemical analysis, using a specific anti-nitrotyrosine antibody, revealed a positive staining in lungs from carrageenan-treated

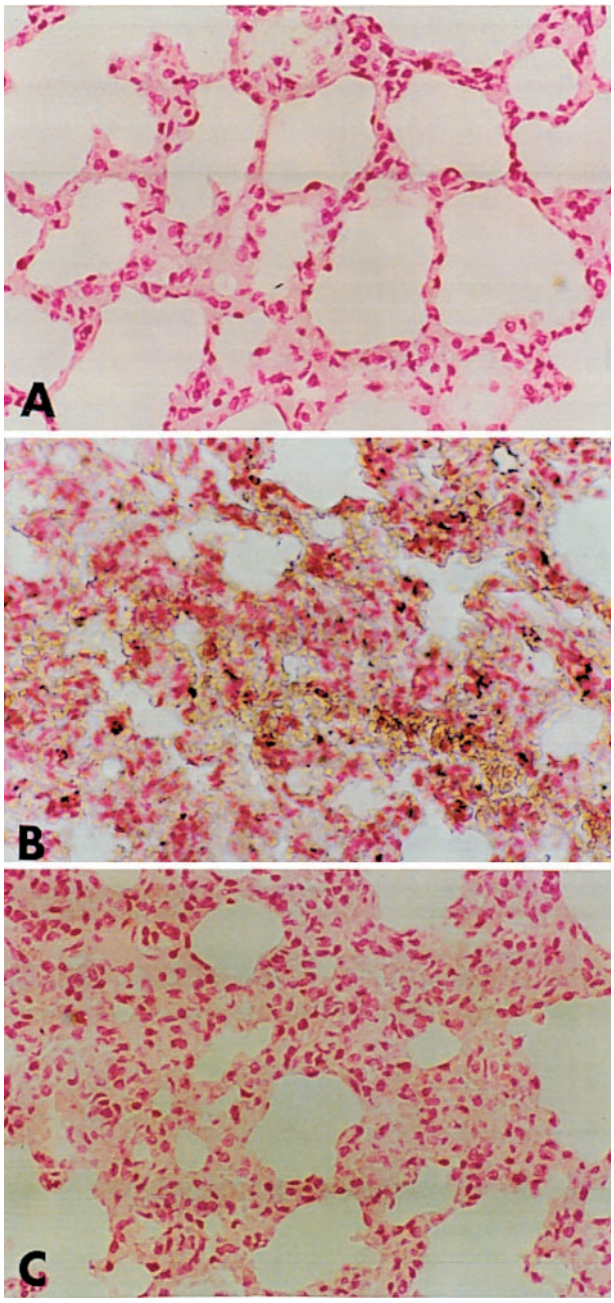

Figure 5 Immunohistochemical localization of nitrotyrosine in the rat lung. Staining was absent in control tissue (A). Four hours following carrageenan injection, nitrotyrosine immunoreactivity was localized mainly to macrophages and some epithelial cells (B). There is a marked reduction in the immunostaining in the lungs of carrageenan-treated rats when rats were pretreated with raxofelast (20 mg kg^{-1}) (C). Original magnification: $\times 125$. Figure is representative of at least three experiments performed on different experimental days.

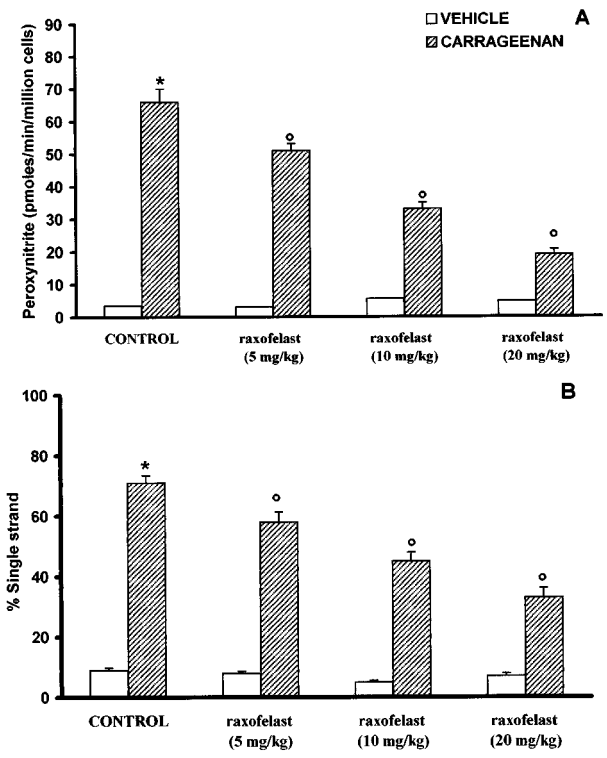

Figure 6 Peroxynitrite formation (A) and DNA single strand breakage development in pleural macrophages harvested from control and carrageenan-treated rats. Four hours after carrageenan injection a significant peroxynitrite production and a marked increase in DNA strand breakage was observed. Raxofelast significantly (5, 10, 20 mg kg^{-1}) inhibited in a dose dependent manner dihydrorhodamine 123 oxidation and prevented the carrageenan-induced DNA single strand breakage. Data represent the number of wells studied (6–9 wells from 2–3 independent experiments). *$P < 0.01$ versus macrophages from control rats; °$P < 0.01$ represents a significant inhibitory effect of raxofelast.

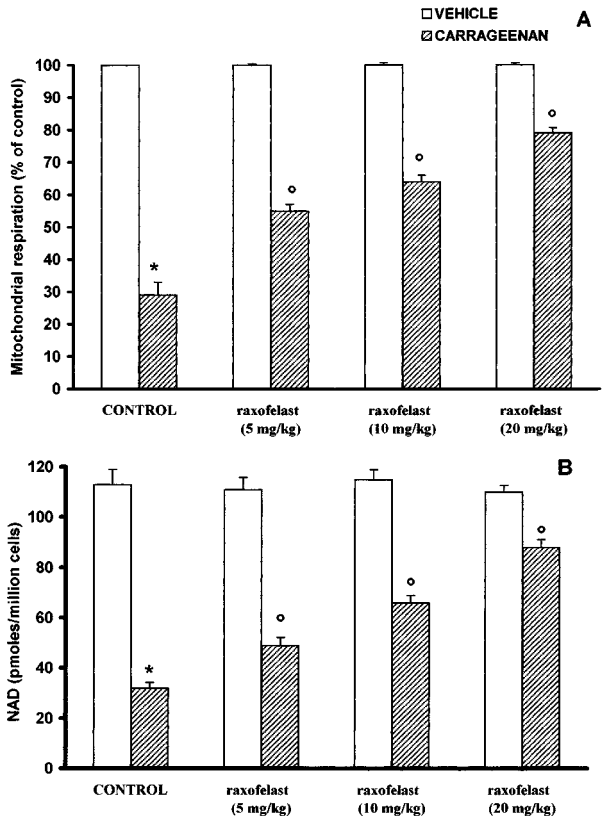

Figure 7 Reduction of mitochondrial respiration (A) and cellular levels of NAD^+ (B) in macrophages from control and carrageenan-treated rats. Four hours after carregeenan administration the disruption of cellular energetic pool was evidenced by a significant decrease in mitochondrial respiration and intracellular concentration of NAD^+. Raxofelast (5, 10, 20 mg kg^{-1}) significantly inhibited in a dose dependent manner the decrease in cellular respiration and partially restored the depletion of intracellular levels of NAD^+. Data represent the number of wells studied (6–9 wells from 2–3 independent experiments). $^*P<0.01$ versus macrophages from control rats; $^\circ P<0.01$ represents protective effects of raxofelast.

rats, which was primarily localized in alveolar macrophages and in airway epithelial cells (Figure 5B). No positive nitrotyrosine staining was found in the lungs of the carrageenan-treated rats when they were treated with raxofelast (Figure 5C).

Raxofelast protects against the cellular energetic failure

In pleural macrophages obtained from carrageenan-treated rats, a significant peroxynitrite production was detectable (66 ± 4 pmoles min^{-1} cells; Figure 6A). A marked increase in DNA strand breakage was also observed after carrageenan-induced pleurisy (Figure 6B). Carregeenan-mediated disruption of cellular energetic pool was evidenced by a significant decrease in mitochondrial respiration and intracellular concentration of NAD^+ (Figure 7A,B). The *in vivo* treatment of the animals with raxofelast significantly inhibited in a dose dependent manner dihydrorhodamine 123 oxidation, prevented the carrageenan-induced DNA single strand breakage (Figure 6A,B), significantly inhibited the decrease in cellular respiration and partially restored the depletion of intracellular levels of NAD^+ (Figure 7A,B).

Discussion

Inflammatory process is invariably characterized by a production of prostaglandins, leukotrienes, histamine, bradykinin, platelet-activing factor (PAF) and interleukin I (IL-1), by a release of chemicals from tissues and migrating cells (Vane & Botting, 1987; Tomlinson *et al*., 1994). Furthermore, one possibility that has been frequently discussed is that production of reactive oxidants such as hydrogen peroxide, superoxide and hydroxyl radical at 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*., 1996a,b; Cuzzocrea *et al*., 1997c; 1998a). Pharmacological inhibitors of NOS have been shown to reduce the development of carrageenan-induced inflammation and support a role of NO in this model of inflammation (Tracey *et al*., 1995; Wei *et al*., 1995; Salvemini *et al*., 1996a,b; Cuzzocrea *et al*., 1997c; 1998a). More recent studies have shown the formation of peroxynitrite in carrageenan-induced inflammation (Salvemini *et al*., 1996a,b; Cuzzocrea *et al*., 1997c; 1998a,b). Using nitrotyrosine immunohistochemistry, this study has confirmed the production of peroxynitrite in the lung of rats subjected to carrageenan-induced pleurisy.

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; Villa *et al*., 1994; Rubbo *et al*., 1994; Pryor & Squadrito, 1996).

In the present study we found that (1) raxofelast reduces the development of carrageenan-induced pleurisy (2) raxofelast reduces morphological injury and neutrophil infiltration in carrageenan-induced inflammation; and (3) raxofelast reduces nitrotyrosine immunostaining, an indicator of peroxynitrite formation in inflammation.

It could be speculated that the pharmacological effects of raxofelast may be dependent upon a combination of: (1) a scavenging action toward oxygen radicals, which results in the prevention of peroxynitrite formation with consequent protection against the development of peroxynitrite-induced cellular energetic failure, (2) a reduced neutrophil recruitment into the inflammatory site, which may represent an important additional mechanism for the observed anti-inflammatory action. The reduced neutrophil infiltration into the inflamed tissue may be related to a prevention of endothelial oxidant injury by raxofelast and to a preservation of endothelial barrier function. Therefore, the relative contribution of the raxofelast's multiple modes of action observed in the current study to be determined in further studies.

Recent studies have proposed that a novel pathway, involving the nuclear enzyme poly (ADP-ribose) synthetase (PARS) play an important role in inflammation (Szabò *et al*., 1997a; 1998; Cuzzocrea *et al*., 1998b,c). It is well known that this pathway plays an important role in various forms of shock (Szabò *et al*., 1997b) and reperfusion injury (Thiemermann *et al*., 1997; Zingarelli *et al*., 1997; Cuzzocrea *et al*., 1997b). Raxofelast has been shown to display potent protective effects against oxidative damage in *in vitro* studies (Mattioli *et al*., 1991) and in various models of oxyradical-mediated ischaemia-reperfusion injury (Campo *et al*., 1992; 1994). The protection by raxofelast against the development of DNA single strand breakage and the partially restoration of intracellular NAD^+ depletion (as shown in Figures 6A and 7B), may thus be related to a decreased peroxynitrite formation, which may thus lead to a prevention of the activation of PARS in inflammation.

Taken together, the results of the present study coupled with recent data by several groups support the view that raxofelast can exert protective effects on cellular injury. In conclusion raxofelast, an α-tocopherol analogue with free radical scavenging properties, reduces the inflammatory response in rats subjected to carrageenan-induced pleurisy. It may therefore have potential therapeutic effects.

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References

BECKMAN, J.S., BECKMAN, T.W., CHEN, J., MARSHALL, P.A. & FREEMAN, B.A. (1990). Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 1620–1624.

CAMPO, G.M., SQUADRITO, F., IOCULANO, M., AVENOSO, A., ZINGARELLI, B., CALANDRA, S., SCURI, R., SAITTA, A. & CAPUTI, A.P. (1992). IRFI 016 a new radical scavenger limits ischemic damage following coronary artery occlusion in rats. *Res. Commun. Chem. Pathol. Pharmacol.,* **76,** 287–303.

CAMPO, G.M., SQUADRITO, F., IOCULANO, M., POLLICINO, A.M., RIZZO, A., CALAPAI, G., CALANDRA, S., SCURI, R. & CAPUTI, A.P. (1994). Protective effect of IRFI 016, a new antioxidant agent, in myocardial damage following coronary artery occlusion and reperfusion in rats. *Pharmacol.,* **48,** 157–166.

CAMPO, G.M., GECCARELLI, S., SQUADRITO, F., ALTAVILLA, D., DORIGOTTI, L. & CAPUTI, A.P. (1997). Raxofelast (IRFI 016): a new hydrophilic vitamin E-like antioxidant agent. *Cardiovasc. Drug Rev.,* **15,** 157–1173.

CECCARELLI, S., DE VELLIS, P., SCURI, R. & ZANARELLA S. (1993). Synthesis of novel 2-substituted-5-oxycoumarans via a direct route to 2,3-dihydro-5-hydroxy-2-benzofuranacetic acids. *J. Heterocycl. Chem.,* **30,** 679–690.

CUZZOCREA, S., ZINGARELLI, B., CALAPAI, G., NAVA, F. & CAPUTI, A.P. (1997a). Zymosan-activated plasma induces paw oedema by nitric oxide and prostaglandin production. *Life Sci.,* **60,** 215–220.

CUZZOCREA, S., ZINGARELLI, B., COSTANTINO, G., SZABÓ, A., SALZMAN, A.L., CAPUTI, A.P. & SZABÓ, C. (1997b). Beneficial effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthetase in a rat model of splanchnic artery occlusion and reperfusion. *Br. J. Pharmacol.,* **121,** 1065–1074.

CUZZOCREA, S., ZINGARELLI, B., GILARD, E., HAKE, P., SALZMAN, A.L. & SZABÓ, C. (1997c). Protective effect of melatonin in carrageenan-induced models of local inflammation. *J. Pineal Res.,* **23,** 106–116.

CUZZOCREA, S., ZINGARELLI, B., GILARD, E., HAKE, P., SALZMAN, A.L. & SZABÓ, C. (1998a). Anti-inflammatory effects of mercaptoethylguanidine, a combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, in carrageenan-induced models of inflammation. *Free Rad. Biol. Med.,* **24,** 450–459.

CUZZOCREA, S., CAPUTI, A.P. & ZINGARELLI, B. (1998b). Peroxynitrite-mediated DNA strand breakage activates poly (ADP-ribose) synthetase and causes cellular energy depletion in carrageenan-induced pleurisy. *Immunology,* **93,** 96–101.

CUZZOCREA, S., ZINGARELLI, B., GILARD, E., HAKE, P., SALZMAN, A.L. & SZABÓ, C. (1998c). Protective effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthase in carrageenan-induced models of local inflammation. *Eur. J. Pharmacol.,* **342,** 67–76.

DA MOTTA, J.I., CUNHA, F.Q., VARGAFTIG, B.B. & FERREIRA, S.H. (1994). Drug modulation of antigen-induced paw oedema in guinea-pigs: effects of lipopolysaccharide, tumour necrosis factor and leucocyte depletion. *Br. J. Pharmacol.,* **112,** 111–116.

DARLEY-USMAR, V. & HALLIWELL, B. (1996). Blood radicals. Reactive nitrogen species, reactive oxygen species, transition metal ions, and the vascular system. *Pharmaceut. Res.,* **13,** 649–655.

DAWSON, J., SEDGWICK, A.D., EDWARDS, J.C. & LEES, P. (1991). A comparative study of the cellular, exudative and histological responses to carrageenan, dextran and zymosan in the mouse. *Int. J. Tissue React.,* **13,** 171–185.

HALLIWELL, B. (1995). Oxygen radicals, nitric oxide and human inflammatory joint disease. *Ann. Rheumat. Dis.,* **54,** 505–510.

HELLER, B., WANG, Z.Q., WAGNER, E.F., RADONS, J., BURKLE, A., FEHSEL, K., BURKART, V. & KOLB, H. (1995). Inactivation of the poly(ADP-ribose) polymerase gene affects oxygen radical and nitric oxide toxicity in islet cells. *J. Biol. Chem.,* **270,** 11176.

MATTOLI, S., MEZZETTI, M., ALLEGRA, L. & BRAGA, P.C. (1991). Dihydro-hydroxy-trimethyl-benzofuranyl acetic acid inhibits lipid peroxydation and lysosomal enzyme release in bronchial epithelial cells and macrophages. *Ital. J. Chest Dis.,* **45,** 78–80.

MCCORD, J. (1993). Oxygen-derived free radicals. *New Horizons,* **1,** 70–76.

MONCADA, S., PALMER, R.M.J. & HIGGS, E.A. (1991). Nitric oxide: Physiology, Pathophysiology and Pharmacology. *Pharmacol. Rev.,* **43,** 109–142.

MULLANE, K.M., WESTLIN, W. & KRAEMER, R. (1988). Activated neutrophils release mediators that may contribute to myocardial injury and dysfunction associated with ischemia and reperfusion. *Ann. N.Y. Acad. Sci. U.S.A.,* **524,** 103–121.

NATHAN, C. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.,* **6,** 3051–3064.

OHKAWA, H., OHISHI, N. & YAGI, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.,* **95,** 351–358.

OH-ISHI, S., HAYASHI, I., HAYASHI, M., YAMAKI, K. & UTSUNOMIYA, I. (1989). Pharmacological demonstration of inflammatory mediators using experimental inflammatory models: rat pleurisy induced by carrageenin and phorbol myristate acetate. *Dermatologica,* **179** (suppl 1), 68–71.

PESKAR, B.M., TRAUTMANN, M., NOWAK, P. & PESKAR, B.A. (1991). Release of 15-hydroxy-5,8,11,13-eicosatetraenoic acid and cysteinyl-leukotrienes in carrageenin-induced inflammation: effect of non-steroidal anti-inflammatory drugs. *Agents Actions,* **33,** 240–246.

PRYOR, W. & SQUADRITO, G. (1995). The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.,* **268,** L699–L772.

RUBBO, H., RADI, R., TRUJILLO, M., TELLERI, R., KALYANARAMAN, B., BARNES, S., KIRK, M. & FREEMAN, B.A. (1994). Nitric oxide regulation of superoxide and peroxynitrite-dependent lipid peroxidation. Formation of novel nitrogen-containing oxidised lipid derivatives. *J. Biol. Chem.,* **269,** 26066–26075.

SALVEMINI, D., WANG, Z.Q., WYATT, P., BOURDON, D.M., MARINO, M.H., MANNING, P.T. & CURRIE, M.G. (1996a). Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.,* **118,** 829–838.

SALVEMINI, D., WANG, Z.Q., BOURDON, D.M., STERN, M.K., CURRIE, M.G. & MANNING, P.T. (1996b). Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. *Eur. J. Pharmacol.,* **303,** 217–220.

SCHRAUFSTATTER, I.U., HINSHAW, D.B., HYSLOP, P.A., SPRAGG, R.G. & COCHRANE, C.G. (1986). Oxidant injury of cells: DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J. Clin. Invest.,* **77,** 1312–1320.

SZABÓ, C. (1996). The role of peroxynitrite in the pathophysiology of shock, inflammation and ischemia-reperfusion injury. *Shock,* **6,** 79–87.

SZABÓ, C., LIM, L.H.K., CUZZOCREA, S., GETTING, S.J., ZINGARELLI, B., FLOWER, R.J., SALZMAN, A.L. & PERRETTI, M. (1997a). Inhibition of poly (ADP-ribose) synthetase exerts anti-inflammatory effects and inhibits neutrophil recruitment. *J. Exp. Med.,* **186,** 1041–1049.

SZABÓ, C., CUZZOCREA, S., ZINGARELLI, B., O'CONNOR, M. & SALZMAN, A.L. (1997b). Endothelial dysfunction in endotoxic shock: importance of the activation of poly (ADP ribose synthetase (PARS) by peroxynitrite. *J. Clin. Invest.,* **100,** 723–735.

SZABÓ, C., VIRÀG, L., CUZZOCREA, S., SCOTT, G.S., HAKE, P., O'CONNOR, M., ZINGARELI, B., MA, Y., HIRSCH, R., BOIOVIN, G.P., SALZMAN, A.L. & KUN, E. (1998). Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly (ADP-Ribose) synthetase. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 3867–3872.

THIEMERMANN, C., BOWES, J., MYNNY, F.P. & VANE, J.R. (1997). Inhibition of the activity of poly(ADP ribose) synthase reduces ischaemia-reperfusion injury in the heart and skeletal muscle. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 679–683.

TOMLINSON, A., APPLETON, I., MOORE, A.R., GILROY, D.W., WILLIS, D., MITCHELL, J.A. & WILLOUGHBY. (1994). Cyclo-oxygenase and nitric oxide isoforms in rat carrageenin-induced pleurisy. *Br. J. Pharmacol.,* **113,** 693–698.

TRACEY, W.R., NAKANE, M., KUK, J., BUDZIK, G., KLINGHOFER, V., HARRIS, R. & CARTER, G. (1995). The nitric oxide synthase inhibitor, L-NG-monomethylarginine, reduces carrageenan-induced pleurisy in the rat. *J. Pharmacol. Exp. Ther.,* **273,** 1295–1299.

VANE, J. & BLOTTING, R. (1987). Inflammation and the mechanism of action of antiinflammatory drugs. *FASEB J.,* **1,** 89–96.

VILLA, L.M., SALAS, E., DARLEY-USMAR, M., RADOMSKI, M.W. & MONCADA, S. (1994). Peroxynitrite induces both vasodilatation and impaired vascular relaxation in the isolated perfused rat heart. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 12383–12387.

WEI, X.Q., CHARLES, I.G., SMITH, A., URE, J., FENG, G.J., HUANG, F.P., XU, D., MULLER, W., MONCADA, S. & LIEW, F.Y. (1995). Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature,* **375,** 408–411.

WIZEMANN, T., GARDNER, C., LASKIN, J., QUINONES, S., DURHAM, S., GOLLER, N., OHNISHI, T. & LASKIN, D. (1994). Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J. Leukoc. Biol.,* **56,** 759–768.

YOUN, Y.K., LALONDE, C. & DEMLING, R. (1991). Use of antioxidant therapy in shock and trauma. *Circ. Shock,* **35,** 245–249.

ZINGARELLI, B., CUZZOCREA, S., ZSENGELLER, Z., SALZMAN, A.L. & SZABÓ, C. (1997). Protection against myocardial ischemia and reperfusion injury by 3-aminobenzamide, an inhibitor of Poly (ADP-Ribose) synthetase. *Cardiovasc. Res.,* **36,** 205–215.

ZINGARELLI, B., O'CONNOR, M., WONG, H., SALZMAN, A.L. & SZABÓ, C. (1996). Peroxynitrite-mediated DNA strand breakage activates poly-adenosine diphosphate ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipolysaccharide. *J. Immunol.,* **156,** 350–358.

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