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European Journal of Pharmacology 342 (1998) 67–76

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Protective effects of 3-aminobenzamide, an inhibitor of poly (ADP-ribose) synthase in a carrageenan-induced model of local inflammation

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Received 12 June 1997; revised 6 October 1997; accepted 10 October 1997

Abstract

A cytotoxic cycle triggered by oxidant-induced DNA single strand breakage and subsequent activation of the nuclear enzyme poly (ADP-ribose) synthetase have been shown to contribute to the cellular injury during various forms of oxidant stress in vitro. The aim of the present study was to investigate the role of poly (ADP-ribose) synthetase in a model of acute local inflammation (carrageenan-induced pleurisy), where oxyradicals, nitric oxide and peroxynitrite are known to play a crucial role in the inflammatory process. The results show that the poly (ADP-ribose) synthetase inhibitor 3-aminobenzamide (given at 1–30 mg/kg) inhibits the inflammatory response (pleural exudate formation, mononuclear cell infiltration, histological injury). Moreover, 3-aminobenzamide reduces the formation of nitrotyrosine, an indicator of the formation of peroxynitrite, in the lung. The present results demonstrate that 3-aminobenzamide, presumably by inhibition of poly (ADP-ribose) synthetase, exerts potent anti-inflammatory effects. Part of the anti-inflammatory effects of 3-aminobenzamide may be related to a reduction of neutrophil recruitment into the inflammatory site. © 1998 Elsevier Science B.V.

Keywords: Nitric oxide $(NO)_i$; Peroxynitrite; Apoptosis; Neutrophil; DNA strand break

1. Introduction

In vitro studies have demonstrated that DNA single strand breakage, which develops in response to various forms of oxidant injury, triggers the activation of the nuclear enzyme poly (ADP-ribose) synthetase, which, in turn, can result in energy depletion and cellular injury (Cochrane, 1991; Szabó et al., 1996a,b). Main triggers of DNA single strand breakage and poly (ADP-ribose) synthetase activation include hydrogen peroxide and hydroxyl radical (Cochrane, 1991) and NO or peroxynitrite (Zhang et al., 1994; Szabó et al., 1996a).

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 roles in the pathogenesis of cell injury in various forms of inflammation (Clancy and Abramson, 1995; Szabó, 1995; Pfeilschifter et al., 1996). NO and superoxide react to form peroxynitrite, a cytotoxic oxidant species (Beckman et al., 1990), which mediates an important part of the cytotoxic effects of peroxynitrite during shock and inflammation (Crow and Beckman, 1995; Beckman and Koppenol, 1996; Szabó, 1996a).

The present work was designed to elucidate whether poly (ADP-ribose) synthetase affects the course of the inflammatory response in a carrageenan-triggered model of local inflammation. In similar experimental models, previous work has demonstrated the anti-inflammatory potential of various therapeutic approaches aimed at scavenging of oxyradicals and at inhibition of NO synthesis and peroxynitrite formation (Tracey et al., 1995; Salvemini et al., 1996a,b; Cuzzocrea et al., 1997a). In our studies, we utilized 3-aminobenzamide, a prototypical pharmacological inhibitor of poly (ADP-ribose) synthetase (Banasik et al., 1992; Szabó et al., 1996a,b). The results of the current study support the view that inhibition of poly (ADP-ribose) synthetase exerts significant anti-inflammatory effects.

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2. Materials and methods

2.1. 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 incisions were closed with a suture and the animals were allowed to recover. At 4 h after the injection of carrageenan, the animals were sacrificed by 100% CO_2 breathing. 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 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. The number of leukocytes in the exudate were suspended in phosphate buffer saline and counted with optical microscope by Burker's chamber after vital Trypan blue stain.

For the pharmacological inhibition of poly (ADP-ribose) synthetase, rats were treated with 3-aminobenzamide (1–30

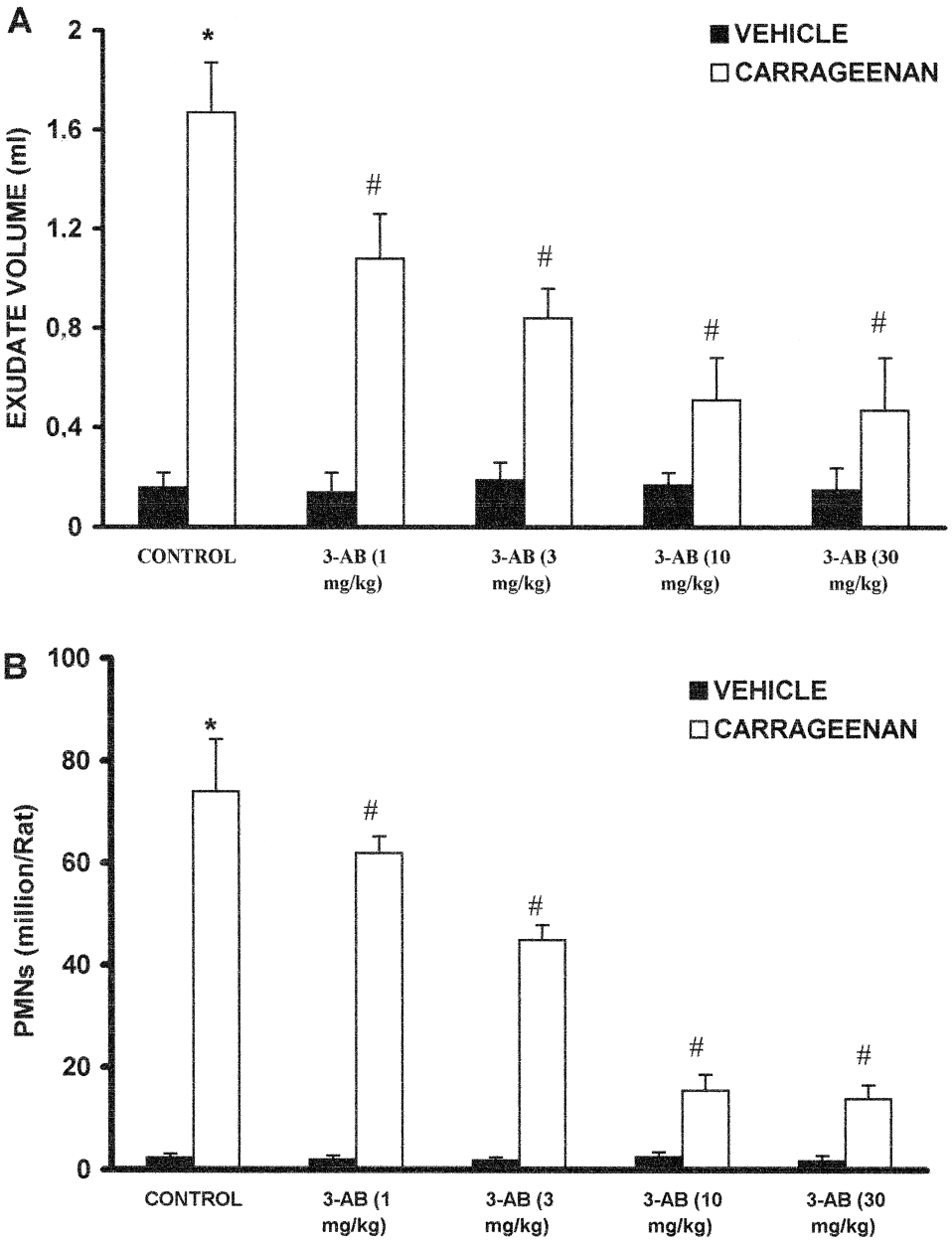


Fig. 1. Exudate volume (A) and polymorphonuclear accumulation (B) in the pleural cavity at 4 h after carrageenan injection. 3-AB (1–30 mg/kg) treatment significantly reduced pleural exudation and leukocyte migration. Data are means ± S.E.M. of 12 rats for each group. $^{*}P < 0.01$ versus sham. $^{\#}P < 0.01$ versus carrageenan.

mg/kg), injected intraperitoneally (i.p.) 1 h after carrageenan in a 0.3 ml volume. Control animals received the same volume of vehicle. Similar doses of 3-aminobenzamide (e.g. 10 mg/kg i.v. or i.p.) have previously been shown to exert protective effects in rat models of endotoxic shock (Szabó et al., 1996b; Szabó et al., 1997a).

2.2. Measurement of nitrite + nitrate concentration in the exudate

Nitrite + nitrate production, an indicator of NO synthesis, was measured in exudate samples as previously described (Zingarelli et al., 1996). First, nitrate in the exudate was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μM) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide 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 a Spectramax 250 microplate reader (Molecular Devices Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

2.3. Histological examination

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

2.4. Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, a specific indicator of peroxynitrite formation, was detected as previously described (Cuzzocrea et al., 1997b) 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% 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 labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories).

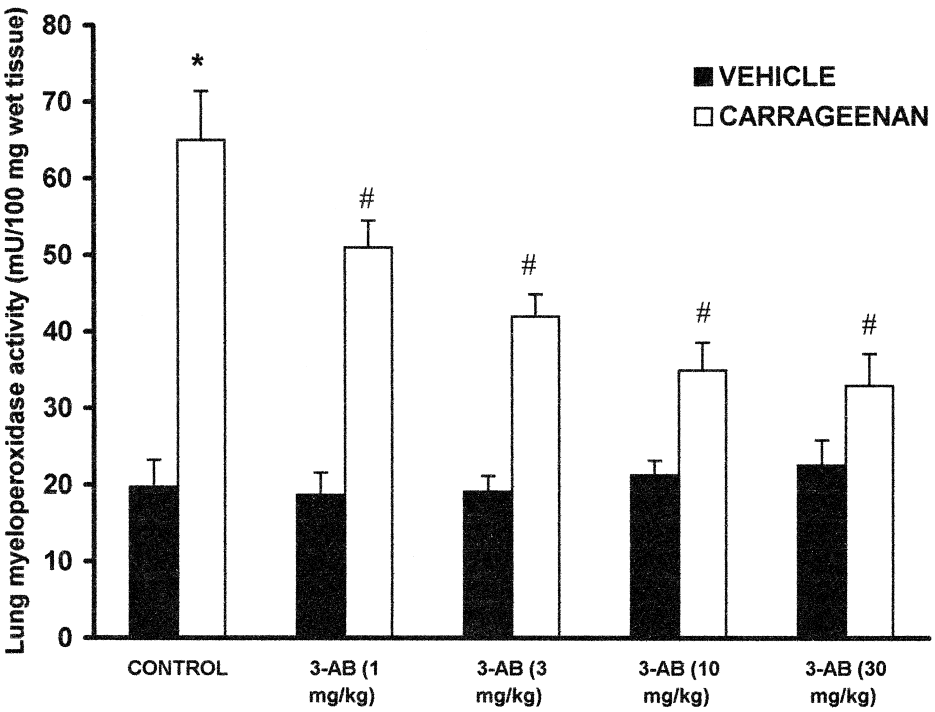


Fig. 2. Myeloperoxidase (MPO) activity in the lungs of carrageenan-treated rats sacrificed at 4 h. MPO activity was significantly increased in the lungs of the carrageenan-treated rats in comparison to sham rats ($^{*}P < 0.01$). 3-AB treatment (1–30 mg/kg) reduced the carrageenan-induced increase in MPO activity. Values are means ± S.E.M. of 12 rats for each group. $^{*}P < 0.01$ vs. sham; $^{\#}P213 < 0.01$ vs. carrageenan.

2.5. Myeloperoxidase activity

Myeloperoxidase activity, an index of polymorphonuclear leukocyte accumulation, was determined as previously described (Mullane et al., 1985). Lungs, 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 of peroxide min^{-1} at 37°C and was expressed in milliunits per gram weight of wet tissue.

2.6. Nitric oxide synthase assay

Lungs, taken 4 h after carrageenan injection, were placed into a homogenization buffer composed of: 50 mM Tris–HCl, 0.1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride (pH 7.4). Tissues were then homogenized in the buffer on ice using a Tissue Tearor 985-370 homogenizer (Biopsies Products, Racine, WI). Conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline was measured in the homogenates as described (Szabó et al., 1994). Briefly, homogenate (30 μl) was incubated in the presence of L-[^{3}H]arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 30 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). Experiments performed in the absence of NADPH determined the extent of L-[^{3}H]citrulline formation independent of a specific NO synthase activity. Experiments in the presence of NADH, without calcium and EGTA (5 mM), determined the calcium-independent (i.e. induced) NO synthase activity. Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted L-[^{3}H]citrulline activity was measured by a scintillation counter (Wallac, Gaithersburg, MD).

2.7. Materials

Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex were obtained from Vector Laboratories (Burlingame, CA). Primary anti-nitrotyrosine antibody was from Upstate Biotech (Saranac Lake, NY). All other reagents and compounds used were obtained from Sigma Chemical Company (Sigma, St. Louis, MO).

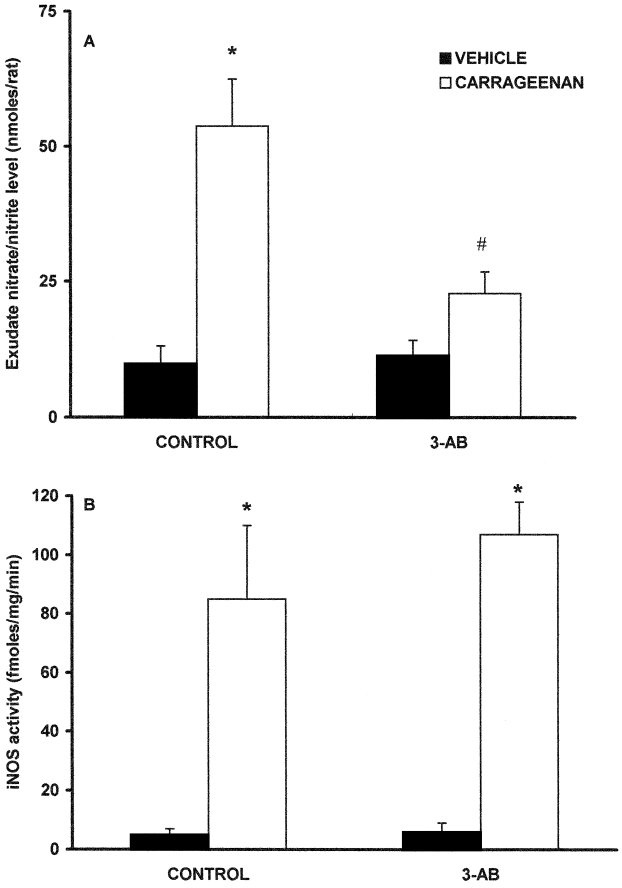
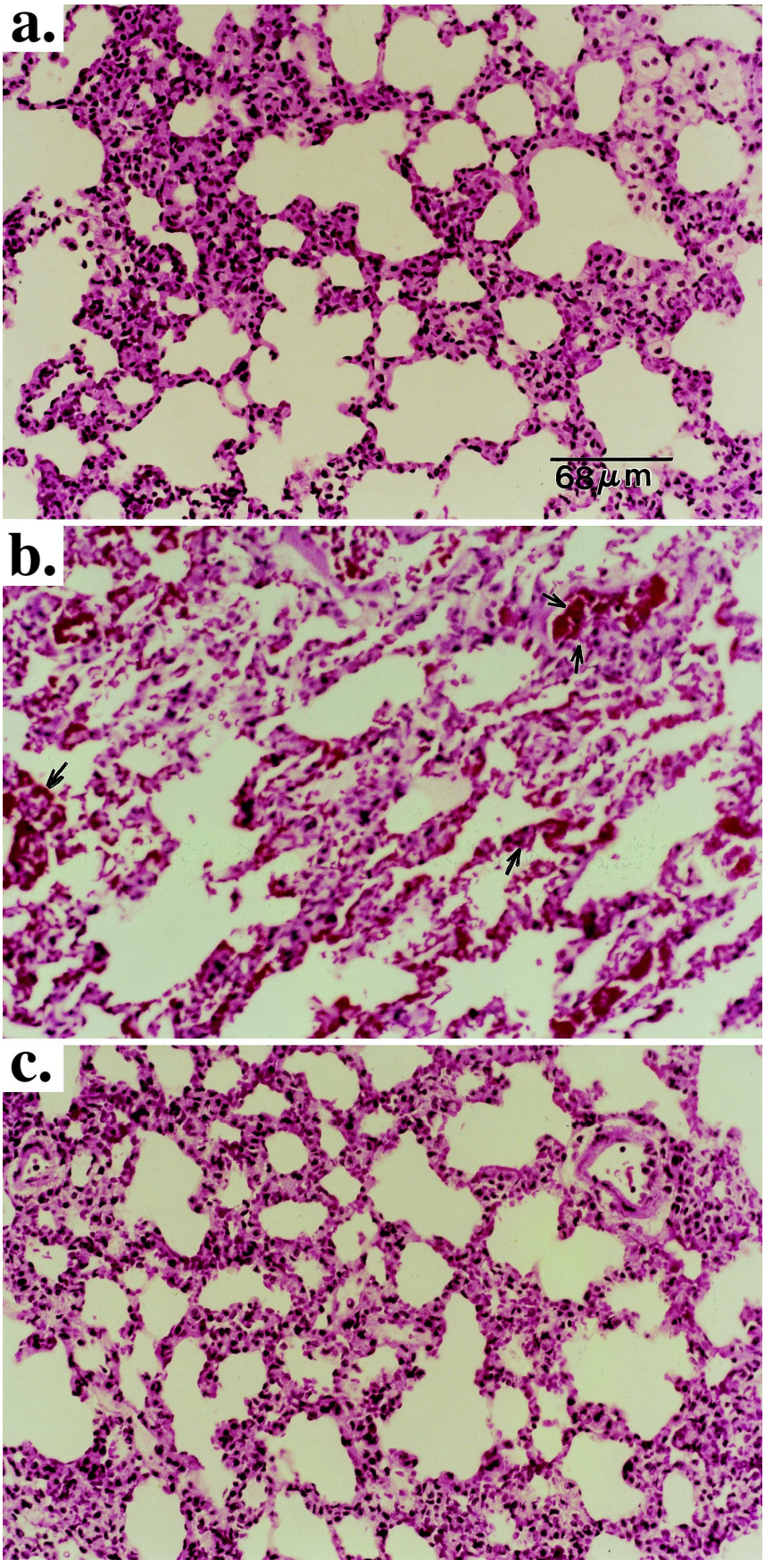


Fig. 3. Nitrite and nitrate level in the pleural exudate (A) and pulmonary iNOS activity (B) at 4 h after carrageenan administration. Nitrite and nitrate levels and iNOS activity in the carrageenan-treated rats were significantly increased versus sham group. 3-AB (10 mg/kg) treatment significantly ameliorated the carrageenan-induced elevation of nitrite and nitrate levels, but did not affect the expression of iNOS activity. Values are means ± S.E.M. of 12 rats for each group. $^*P < 0.01$ versus sham. $^\#P < 0.01$ versus carrageenan.

2.8. 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. Individual group means were then compared with Dunnett's test. A *P*-value less than 0.05 was considered significant. In the experiments involving histology or immunohistochemistry, the figures shown are representative

Fig. 4. Representative lung sections from (A) sham rats demonstrating the normal alveolar architecture. Lung sections from a carrageenan-treated rat (B) demonstrate interstitial hemorrhage and mononuclear cell accumulation (arrows). Lung sections from a carrageenan-treated rat that received 3-AB (10 mg/kg) demonstrate reduced interstitial hemorrhage and a lesser cellular infiltration (C).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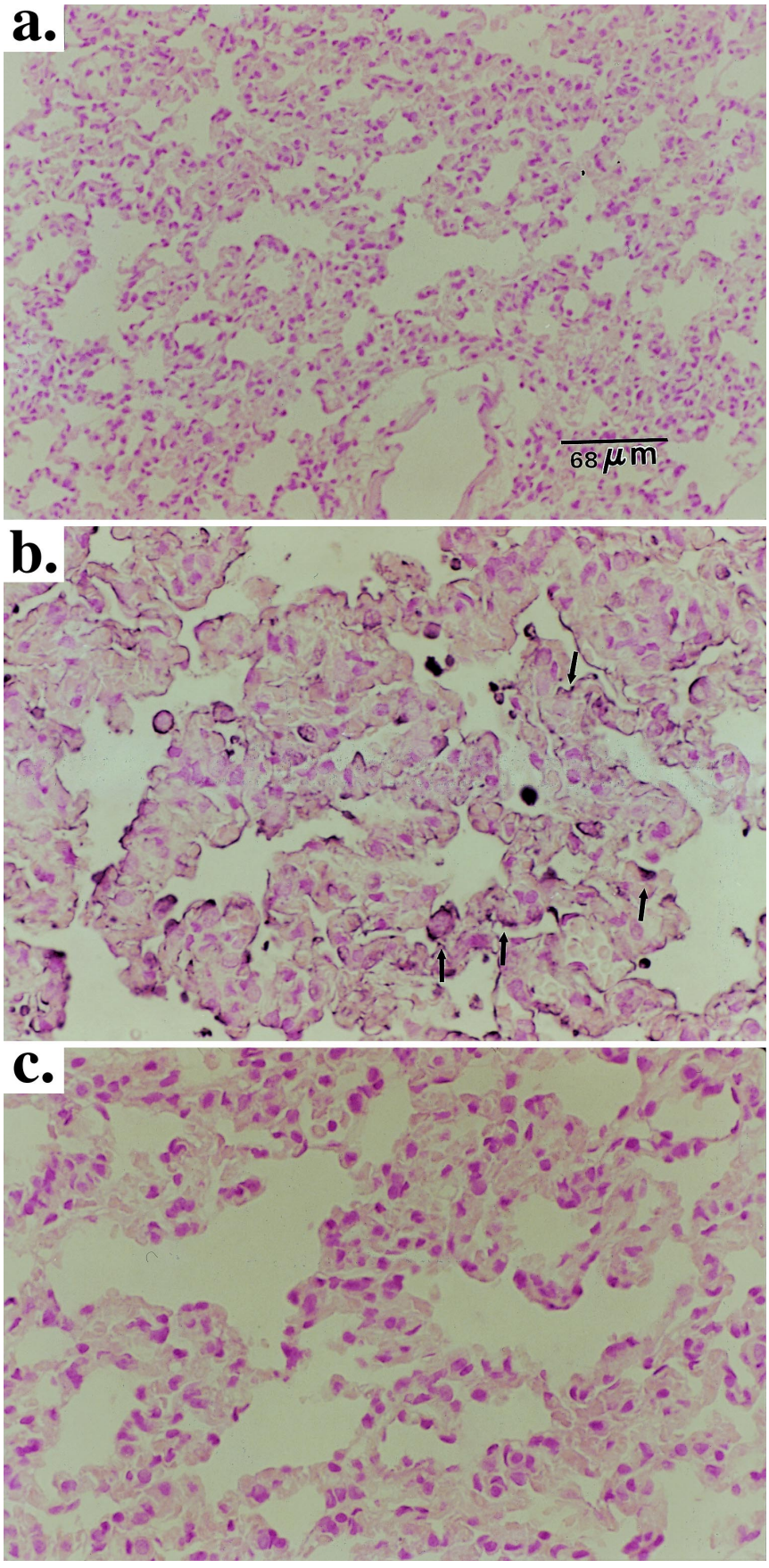
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of at least 3 experiments performed on different experimental days.

3. Results

3.1. Effects of 3-aminobenzamide on the development of carrageenan-induced pleurisy

All carrageenan-injected rats developed an acute pleurisy, as indicated by the production of turbid exudate (Fig. 1A). Trypan blue stain revealed a significant increase in the polymorphonuclear leukocytes in comparison to sham rats (Fig. 1B). Furthermore, there was a significant increase in lung myeloperoxidase activity in response to carrageenan administration, indicative of PMN infiltration into the inflamed tissue (Fig. 2). At the doses of 1–10 mg/kg, there was a dose-dependent relationship between the dose of 3-aminobenzamide and the degree of inhibition of extravasation and PMN accumulation. However, a further increase in the dose of 3-aminobenzamide from 10 to 30 mg/kg did not result in an increase in the anti-inflammatory effects (Figs. 1 and 2). Therefore, the dose of 10 mg/kg of 3-aminobenzamide was selected for further histological and immunohistochemical studies.

Nitrite/nitrate concentrations were also significantly increased in the exudate after carrageenan challenge (Fig. 3A). Sham animals demonstrated no abnormalities in the pleural cavity or fluid. Nitrite/nitrate levels were significantly reduced in rats treated with 3-aminobenzamide (Fig. 3A). 3-Aminobenzamide treatment did not cause significant changes in any of the parameters studied in sham rats (Figs. 1–3).

In the lungs obtained from animals subjected to carrageenan-induced pleurisy, a significant increase of iNOS activity was detected, as measured at 4 h (Fig. 3B). The inducible NO synthase activity was unaffected by 3-aminobenzamide (Fig. 3B).

At 4 h after carrageenan administration, lungs were examined for tissue damage by histological examination. Histological examination revealed extravasation of red cells, and neutrophil and macrophage accumulation after carrageenan exposure (Fig. 4). Treatment with 3-aminobenzamide (10 mg/kg) reduced histological organ injury (Fig. 4).

At 4 h following the intrapleural injection of carrageenan, lung sections were also analyzed 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 rats, which was primarily localised in alveolar macrophages and in airway epithelial cells (Fig. 5). A marked reduction in nitrotyrosine staining was found in the lungs of the carrageenan-treated rats treated with 10 mg/kg 3-aminobenzamide (Fig. 5).

4. Discussion

The main findings of the current study are the following: (1) 3-aminobenzamide dose-dependently reduces morphological injury and neutrophil infiltration in carrageenan-induced models of local inflammation; and (2) 3-aminobenzamide reduces nitrotyrosine immunostaining, an indicator of peroxynitrite formation in inflammation.

The cellular and molecular mechanism of the carrageenan-induced inflammation are well characterized, and these models of inflammation are standard models of screening for anti-inflammatory activity of various experimental compounds. The early phase of the carrageenan edema is related to the production of histamine, leukotrienes, platelet-activating factor, and possibly cyclooxygenase products, while the delayed phase of the carrageenan-induced inflammatory response 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 (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., 1997a). Based on in vitro studies, hydroxyl radical and peroxynitrite are the two most likely triggers of DNA single strand breakage and poly (ADP-ribose) synthetase activation (Cochrane, 1991; Szabó, 1996b).

In a variety of in vitro studies in fibroblasts, endothelial cells, epithelial cells, smooth muscle cells and other cell types, it has been established that pharmacological inhibitors of poly (ADP-ribose) synthetase, such as 3-aminobenzamide, protect against the cellular oxidant injury in response to oxyradicals, NO generator drugs and peroxynitrite (Cochrane, 1991; Szabó, 1996b). Based on the present in vivo studies alone, it is not possible to identify the cell type on which 3-aminobenzamide exerts its protection against oxidant injury during inflammation. Nevertheless, it is noteworthy that a marked inhibition by 3-aminobenzamide of polymorphonuclear leukocyte infiltration into the inflammatory site was observed. Extravasated polymorphonuclear leukocytes in the inflammatory sites become activated and produce a variety of inflammatory mediators such as growth factors, chemokines and cytokines, complement components, proteases, NO, reactive

Fig. 5. Immunohistochemical localization of nitrotyrosine in the rat lung. Staining was absent in control tissue (A). Four h following carrageenan injection, nitrotyrosine immunoreactivity (typical areas are indicated by arrows) was localized mainly to macrophages and some epithelial cells (B). There was a marked reduction in the immunostaining in the lungs of carrageenan-treated rats when rats were pretreated with 3-AB (10 mg/kg) (C).

oxygen metabolites and peroxynitrite, which are important mediators of tissue injury (Fujishima and Aikawa, 1995). Prevention of the activation of neutrophil-dependent inflammatory pathways is likely to contribute to the reduced fluid extravasation and improved histological status after inhibition of poly (ADP-ribose) synthetase.

Thus, we propose that 3-aminobenzamide may exert anti-inflammatory effects by two distinct mechanisms. First, extensive activation of poly (ADP-ribose) synthetase due to massive oxidant-mediated DNA injury can lead to pronounced NAD^+ and ATP depletion in various tissues, and 3-aminobenzamide may reduce this injury. Second, a reduced neutrophil recruitment may represent an important additional mechanism for the anti-inflammatory effects provided by inhibition of poly (ADP-ribose) synthetase (Szabó et al., 1997b). The mechanism of this reduced neutrophil recruitment is not clear at present. It may be related to a prevention by 3-aminobenzamide of endothelial oxidant injury (Kirkland, 1991; Szabó et al., 1997a). Alternatively, it may be related to inhibition by 3-aminobenzamide of the expression of adhesion receptors in inflammation (Hiromatsu et al., 1993) and/or to inhibition of the neutrophil-endothelial interaction (Meyer et al., 1991). An additional mechanism which may be considered as a contributor to the anti-inflammatory effects of 3-aminobenzamide may be related to inhibition of the expression of the inducible NO synthase by this agent. Indeed, a variety of poly (ADP-ribose) synthetase inhibitors, including 3-aminobenzamide and nicotinamide have been shown to inhibit the process of iNOS expression, although the concentrations required for this effect were rather high (Pellat-Seceunyk et al., 1994; Zingarelli et al., 1996; Szabó et al., 1997c). However, in the present study, we have found that 3-aminobenzamide, given as a 1 h post-treatment, did not alter the expression of the inducible NO synthase in the lung, as measured in whole lung homogenates. Similarly, 3-aminobenzamide, given in a similar treatment regimen during endotoxic shock, did not affect the process of the expression of the inducible NO synthase (Szabó et al., 1996a, 1997c).

Perhaps somewhat surprisingly, 3-aminobenzamide inhibited nitrite/nitrate concentrations in the pleural exudate, and reduced nitrotyrosine staining in the lungs, despite the fact that it did not inhibit the expression of the inducible NO synthase. Since 3-aminobenzamide is not a scavenger of NO or peroxynitrite, nor does the agent inhibit NO synthase (Zhang et al., 1994; Zingarelli et al., 1996, 1997b), the most likely explanation for this finding is that the reduction in the nitrite/nitrate concentrations in the pleural exudate and the reduced nitrotyrosine staining in the 3-aminobenzamide-treated animals is more likely to be related to the reduced mononuclear cell infiltration (and, subsequently, reduced NO and superoxide production), rather than to an inhibitory effect on NO synthase expression or activity. In conjunction with our finding that 3-aminobenzamide reduces myeloperoxidase content in the lung, it is noteworthy that tyrosine nitration can also be induced by the reaction of myeloperoxidase with nitrite in the presence of hydrogen peroxide (Van der Vliet et al., 1997).

As with most pharmacological inhibitors, we cannot exclude the possibility that additional, poly (ADP-ribose) synthetase-independent effects may contribute to the anti-inflammatory effects observed with 3-aminobenzamide in the current study. For instance, it is unclear at present whether the inhibition of collagenase expression (Ehrlich et al., 1995) or inhibition of adhesion molecule expression (Hiromatsu et al., 1993) are related to inhibition of poly (ADP-ribose) synthetase, or due to unrelated pharmacological effects of poly (ADP-ribose) synthetase inhibitors. A genetically engineered animal lacking poly (ADP-ribose) synthetase has became available recently (Wang et al., 1995). Cells obtained from these animals are resistant to various types of acute oxidant injury (Heller et al., 1995; Szabó et al., 1997a), although they do not appear to be resistant to the delayed, programmed cell death (apoptosis) (Leist et al., 1997). Clearly, the examination of the inflammatory response in the poly (ADP-ribose) synthetase knockout animals will more definitively determine the role of poly (ADP-ribose) synthetase in the inflammatory process. Indeed, we have recently observed that poly (ADP-ribose) synthetase knockout mice are resistant to zymosan-induced multiple organ failure and have reduced neutrophil infiltration into inflamed tissues, when compared to the response in the wild-type counterparts (Szabó et al., 1997b).

Recent investigations have concluded that inhibition of poly (ADP-ribose) synthetase exerts beneficial effects in endotoxic shock (Szabó et al., 1996b) and various forms of reperfusion injury (Zhang and Steiner, 1995; Cuzzocrea et al., 1997a; Thiemermann et al., 1997; Zingarelli et al., 1997a,b). Based on the studies described in the present paper, we propose a role for poly (ADP-ribose) synthetase in the process of the local inflammatory response, and put forward the hypothesis that inhibition of poly (ADP-ribose) synthetase represents a novel anti-inflammatory strategy. The potential risk of this strategy is reduced by the demonstration that cells from the poly (ADP-ribose) synthetase knockout mice do not have a compromised DNA repair, although poly (ADP-ribose) synthetase is traditionally being viewed as an enzyme which is important for the DNA repair processes (Wang et al., 1995). Further studies are needed to compare the efficacy of poly (ADP-ribose) synthetase inhibition with that of other, established or experimental anti-inflammatory approaches.

Acknowledgements

This study was supported by a Grant from the National Institutes of Health (R29GM54773) to C.S.

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