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Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly (ADP-ribose) polymerase, in a rodent model of lung injury

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Abstract

Poly (ADP-ribose) polymerase (PARP), a nuclear enzyme activated by strand breaks in DNA, plays an important role in the tissue injury associated with ischaemia–reperfusion injury and inflammation. The aim of the present study was to evaluate the effects of a novel and potent inhibitor of PARP activity on neutrophil recruitment in the acute inflammation induced by zymosan-activated plasma. Intrathoracic administration of zymosan-activated plasma leads to an increase in neutrophil infiltration of the lung at 24 hr. The potent PARP inhibitor 5-aminoisoquinolinone (5-AIQ) reduced the degree of lung injury and attenuated the expression of P-selectin and ICAM-1 as well as the recruitment of neutrophils into the injured lung. The up-regulation/expression of P-selectin and ICAM-1 in human endothelial cells exposed to oxidative stress (peroxynitrite) or to a pro-inflammatory cytokine (tumor necrosis factor α, TNFα) was also attenuated by 5-AIQ. These findings provide the first evidence that the activation of PARS participates in neutrophil-mediated lung injury by regulating the expression of P-selectin and ICAM-1.

Keywords: 5-Aminoisoquinolinone; Pleurisy; Oxygen radicals; Poly (ADP-ribose) synthetase; Zymosan-activated plasma; Complement

1. Introduction

Acute inflammation is characterized by the immediate infiltration of a specific site or lesion with polymorphonuclear neutrophils (PMNs), followed by monocytes and finally lymphocytes. In the last few years, we have gained substantial insight into the importance of specific adhesion molecules and mediators in the following processes which finally result in the recruitment of PMNs at a specific site of inflammation: (1) margination, capture and rolling on the vascular endothelium; (2) activation and firm adhesion; (3) diapedesis through gaps between endothelial cells in post-capillary venules and (4) finally migration along a gradient of chemokines. Activated PMNs, therefore, play a crucial role in the destruction of foreign antigens and the breakdown and remodeling of injured tissue. The inflammatory process is associated with the formation of many mediators including prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF) and the pro-inflammatory cytokines including interleukin-1 (IL-1), IL-8 and TNFα, to name but a few [1–6]. Leukocyte–endothelial interactions involve a complex interplay among adhesion glycoproteins (i.e. integrins, members of the immunoglobulin superfamily and selectins). One member of the selectin family, P-selectin, is rapidly translocated from the Weibel–Palade bodies to the endothelial cell surface upon activation of endothelial cells with thrombin, histamine, hypoxia-reoxygenation, or oxygen-derived free radicals [7,8]. P-selectin promotes rolling of leukocytes on the endothelium. The rolling of leukocytes is the first step in the interactions of leukocytes with the endothelium and

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Abbreviations: 5-AIQ, 5-aminoisoquinolinone; ATP, adenosine triphosphate; NAD, nicotinamide adenine dinucleotide; NO, nitric oxide; PMNs, polymorphonuclear neutrophils; PARP, poly (ADP-ribose) polymerase; PARS, poly (ADP-ribose) synthetase; ROS, reactive oxygen species; TNFα, tumor necrosis factor α.

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facilitates the activation and adherence of PMNs [7,9]. The firm adhesion of PMNs to the endothelium, however, is a complex phenomenon, which also involves other endothelium-based adhesion molecules. In fact, endothelial adhesion molecules are considered to play a pivotal role in the localization and development of an inflammatory reaction [10]. Intercellular adhesion molecules (ICAM-1) is an adhesion molecule normally expressed at a low basal level, but its expression can be enhanced by various inflammatory mediators such as IL-1 and TNFα [11]. One possible pathway of oxidant-induced injury involves the nuclear enzyme PARP. Activation of PARP is triggered by single strand breaks in DNA and subsequently catalyses the transfer of ADP-ribose moieties from NAD^+ to various nuclear proteins including histones and PARP (automodification domain) itself [12]. Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD^+ and subsequently ATP, which may ultimately cause cell death [13–16]. Radicals including superoxide anions, hydrogen peroxide or hydroxyl radicals cause strand breaks in DNA, activation of PARP and depletion of NAD^+ and ATP in cultured [14–16]. Production of reactive oxidants such as hydrogen peroxide, superoxide and hydroxyl radical at site of inflammation contributes of tissue damage [17–21], strand breaks in DNA [22] and ultimately PARP activation. Inhibitors of PARP activity including 3-aminobenzamide (3-AB) reduce the development of acute [23] and chronic inflammation [24]. However, 3-AB is a weak inhibitor of PARP activity that does not readily cross cell membranes [25,26]. Although, 1,5-dihydroxyisoquinoline and 3,4-dihydro-5-[4-(piperidin-1-yl)butoxy]-*iso*-quinolin-1(2*H*)-one (DPQ) are more potent inhibitors of PARP activity, these agents have to be dissolved in dimethylsulfoxide (DMSO). DMSO itself is a potent scavenger of hydroxyl radicals and inhibits PARP activity. Thus, there is still a great need for the development of potent, water-soluble inhibitors of PARP activity. We have recently reported on a novel route for the synthesis of 5-aminoisoquinoline (5-AIQ) and demonstrated that this compound is a water-soluble, potent inhibitor of PARP activity in human cells [27]. In this study, we report that 5-AIQ reduces the up-regulation expression of P-selectin and ICAM-1 on human endothelial cells and diminishes the neutrophil infiltration into the injured lung. The present data demonstrated a novel mechanism whereby PARP inhibition exerts beneficial effects in acute inflammation.

2. Materials and methods

2.1. Animals

Male BALB/c mice (20–22 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. Preparation of zymosan-activated plasma (ZAP)

ZAP was used as a source of the neutrophil chemotactic factor C5a des-Arg [28]. ZAP was prepared by incubating heparinized (100 IU/mL) plasma obtained from naive rats with zymosan (50 mg/mL) for 1 hr at 37°. Zymosan was then removed by centrifugation (15 min at 3000 rpm). Activated plasma was stored in aliquots at −20°.

2.3. Pleurisy

Pleurisy was induced as described by Henriques *et al.* [29], whereby an adapted needle was inserted into the right side of the thoracic cavity of naive animals to enable the intra-thoracic administration of ZAP (50 μL of a 30% solution). Animals were sacrificed by CO_2 inhalation 24 hr after the stimulation, and their thoracic cavities were opened and flushed with 1 mL of heparinized RPMI-1640 (20 IU/mL). The amount of exudate was calculated by subtracting the volume injected (1 mL) from the total volume recovered. The total leukocytes counts were performed in a Burker's chamber after vital Trypan Blue staining.

2.4. Experimental groups

In the treated group of animals, 5-AIQ (3 mg/kg) was given as an intraperitoneal (i.p.) bolus 1 and 6 hr after ZAP (ZAP + 5-AIQ group). In the vehicle-treated group of mice, vehicle (saline) was given instead of 5-AIQ (ZAP group). In separate groups, surgery was performed identically in every aspect to the one in the ZAP group, except that plasma, which had not been activated with zymosan, was injected instead of ZAP (Sham group; Sham). In an additional group of animals, Sham surgery was combined with the administration of 5-AIQ (dose) (Sham + ZAP).

2.5. Measurement of cytokines

TNFα and IL-1β levels were evaluated in the exudate at 24 hr after the induction of pleurisy by ZAP injection. The assay was carried out by using a colorimetric, commercial ELISA kit (Calbiochem-Novabiochem Corporation, USA).

2.6. Histological examination

Lung biopsies were taken at 24 hr after injection of ZAP. The biopsies were fixed for 1 week in buffered formaldehyde solution (10% in PBS) at room temperature, dehy-

drated 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).

2.7. Immunohistochemical localization of ICAM-1, P-selectin and PARP

Immunohistochemical staining was performed on 7 μm thick sections of unfixed mice lung. Sections were cut in with a Slee and London cryostat at −30°, transferred onto clean glass slides and dried overnight at room temperature. Sections were permeabilized with acetone at −20° for 10 min and rehydrated in phosphate buffered saline (PBS) (150 mM NaCI, 20 mM sodium phosphate pH 7.2) at room temperature for 45 min. Sections were incubated overnight with (1) monoclonal biotinylated antibodies directed at P-selectin (rat anti-mouse CD62P) or ICAM-1 (hamster anti-mouse CD54) at a dilution 1:500 in PBS (v/v) or (2) with anti-poly (ADP-ribose) monoclonal antibody (1:500 in PBS (v/v)). Sections were washed with PBS, and incubated with secondary antibody for 2 hr at room temperature. Specific labeling was detected with a avidin–biotin peroxidase complex.

2.8. Measurement of lung-tissue myeloperoxidase activity and malondialdehyde

Myeloperoxidase (MPO) activity, a haemoprotein located in azurophil granules of neutrophils, has been used as a biochemical marker for neutrophil infiltration into tissues [30]. In the present study, MPO was measured photometrically by a method similar to that described previously [31]. At 4 hr following the intrapleural injection of ZAP, lung tissues were obtained and weighed. Each piece of tissue was homogenized in a solution containing 0.5% (w/v) 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°. An aliquot of the supernatant was then allowed to react with a solution of tetramethylbenzidine (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 per minute at 37° and was expressed in milliunits per 100 mg weight of wet tissue. Malondialdehyde (MDA) levels in the lung tissue were determined as an indicator of lipid peroxidation [32]. Lung tissues, collected at the specified time, were homogenized in 1.15% (w/v) KCl solution. An aliquot (100 μL) of the homogenate was added to a reaction mixture containing 200 μL of 8.1% (w/v v/v) SDS, 1500 μL of 20% (v/v) acetic acid (pH 3.5), 1500 μL of 0.8% (w/v) thiobarbituric acid and 700 μL distilled water. Samples were then boiled for 1 hr at 95° and centrifuged at 3000 *g* for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.9. Cell culture

Human umbilical vein endothelial cells (BioWhittaker) were cultured in endothelial growth medium Bullet Kit w/2% FBS (BioWhittaker). Cells were cultured in 96-well plates (200 μL medium per well) or in 12-well plates (3 μL medium per well) until 90% confluence. Cells were exposed to peroxynitrite (100 μmol/L) or TNFα (100 unit/mL) the presence or absence of the PARP inhibitor 5-AIQ (0.003–1 mM).

2.10. Measurement of cell viability

Cell energetic status was assessed by measuring the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan [20]. Cells in 96-well plates were incubated at 37° with MTT (0.2 mg/mL) for 1 hr. Culture medium was removed by aspiration and the cells were dissolved in DMSO (100 μL). The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. As previously discussed [20], the measurement of reduction of MTT appears to be mainly by 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.

2.11. Measurement of cellular NAD^+ levels in pleural macrophages

We measured NAD^+ levels as a mean to indirectly evaluate PARP activation as previously done by others [15,22,33]. Cells in 12-well plates were extracted in 0.25 mL of 0.5 N $HClO_4$ scraped, neutralized with 3 M KOH, and centrifuged for 2 min at 10,000 *g*. The supernatant was assayed for NAD^+ using a modification of the colorimetric method [34,35] in which NADH produced by enzymatic cycling with alcohol dehydrogenase, reduces MTT to formazan through the intermediation of phenazine 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 MTT, 20 μL of a solution of 4 mg/mL phenazine methosulfate, 10 μL of a solution of 0.6 mg/mL alcohol dehydrogenase (300 unit/mg), 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° 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° against blank at 560 nm in the ELISA microplate reader (SLT-Labinstruments Salzburg, Austria).

2.12. ELISA for P-selectin and ICAM-1 expression

The expression of P-selectin and ICAM-1 was evaluated in HUVECs by an ELISA method (Burke-Gaffney & Hellewell, 1996). Briefly, after stimulation with peroxynitrite or TNFα, cells in 96-well plates were washed with HBSS (pH 7.4), fixed with 1% paraformaldehyde for 15 min, and incubated for 1 hr with a 2% BSA-HBSS solution. The primary monoclonal antibodies directed at P-selectin or ICAM-1 (1:700 dilution) were then added for 2 hr. Thereafter, a secondary developing antibody (peroxidase conjugate) was applied for 1 hr followed by incubation with the substrate 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (1 mg/mL in 0.2 mol/L citrate/phosphate buffer, pH 5, containing 0.1% hydrogen peroxide) for 30 min. All the incubations were carried out at room temperature. Chromophore development was determined by measuring optical density at 405 nm with a Spetramax microplate reader. Background absorbance was determined from cells incubated without primary antibodies.

2.13. Materials

Cell culture medium, heparin and fetal calf serum were obtained from Sigma. Perchloric acid was obtained from Aldrich. Primary monoclonal P-selectin (CD62P) or ICAM-1 (CD54) for immunohistochemistry were purchases by Pharmingen. The primary monoclonal antibodies directed at P-selectin or ICAM-1 for ELISA were obtained from R&D system. Reagents and secondary and nonspecific IgG antibody for immunohistochemical analysis were from Vector Laboratories Inc. Primary monoclonal anti-poly (ADP-ribose) antibody was purchased by Alexis. All other reagents and compounds used were obtained from Sigma Chemical Company.

2.14. Data analysis

All values in the figures and text are expressed as mean ± SEM 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 analyzed by 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 5-AIQ in ZAP-induced pleurisy

Histological examination of lung sections revealed significant tissue damage (Fig. 1B). Thus, when compared to lung sections taken from saline-treated animals (Fig. 1A), histological examination of lung sections of mice treated with ZAP showed edema, tissue injury as well as infiltration of the tissue with neutrophils (PMNs) (Fig. 1B). 5-AIQ significantly reduced the degree of tissue injury as well as the infiltration of PMNs. Furthermore, the injection of ZAP into the pleural cavity of mice elicited an acute inflammatory response characterized by the accumulation of PMNs (Fig. 2A and B). Neutrophils also infiltrated in the lung tissues (Fig. 2B) and this was associated with lipid peroxidation of lung tissues as evidenced by an increase in the levels of malonyldialdehyde (Fig. 3). Edema, neutrophil infiltration in lung tissue, and lipid peroxidation were attenuated by the intraperitoneal injection of 5-AIQ (Figs. 2 and 3).

3.2. Effects of 5-AIQ on the up-regulation expression of adhesion molecules (ICAM-1, P-selectin)

Staining of lung tissue sections obtained from saline-treated mice with anti-ICAM-1 antibody showed a specific staining along bronchial epithelium demonstrating that ICAM-1 is constitutively expressed (Fig. 4A). No positive staining for P-selectin was found in lung tissue section from saline-treated rats (Fig. 5A). At 24 hr after ZAP injection, the staining intensity substantially increased along the bronchial epithelium (Fig. 4B). Lung tissue sections obtained from ZAP-treated mice showed positive staining for P-selectin localized in the bronchial epithelium (Fig. 5B). Less positive staining for ICAM-1 (Fig. 4C) and no positive staining for P-selectin (Fig. 5C) was found in the lungs of ZAP-treated mice that received intraperitoneal injection of 5-AIQ. To verify the binding specificity for ICAM-1 or P-selectin, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

3.3. Effects of 5-AIQ on PARP activity

At 24 hr after ZAP injection, lung sections were taken in order to determine the immunohistological staining for poly ADP-ribosylated proteins (an indicator of PARP activation). Sections of lung from saline-treated mice did not reveal any positive staining for poly ADP-ribosylated proteins (Fig. 6A) within the normal architecture. A positive staining for the PARP polymer (Fig. 6B) was found primarily localized in the vessels and in the

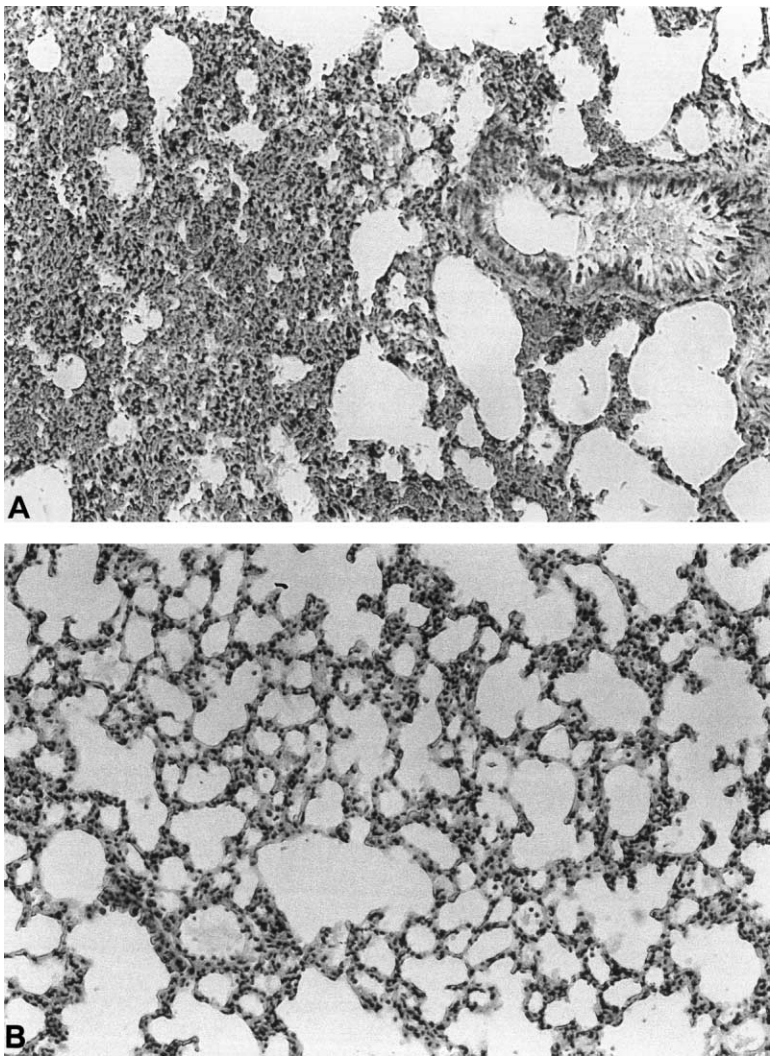

Fig. 1. Effect of 5-AIQ: lung sections from ZAP-treated mice (A) demonstrates interstitial hemorrhage and polymorphonuclear leukocyte accumulation. Lung sections from a ZAP-treated mice that had received 5-AIQ (3 mg/kg, intraperitoneally) (B) exhibit reduced interstitial hemorrhage and a lesser cellular infiltration. Original magnification: 62.5×. Representative of at least three experiments performed on different experimental days.

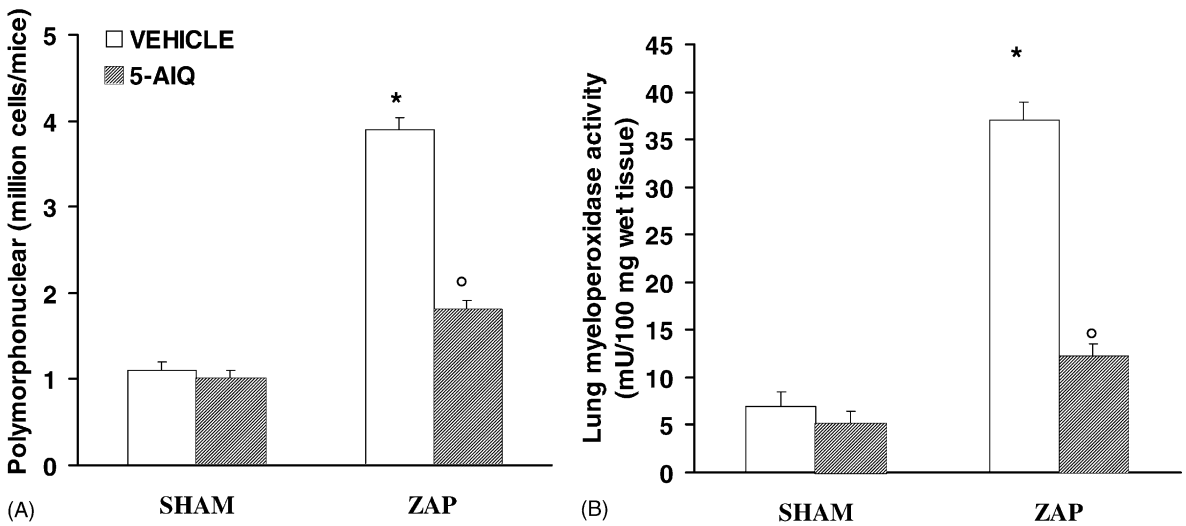

Fig. 2. Effect of 5-AIQ, on ZAP-induced inflammatory cells infiltration: the accumulation of PMNs (A) in pleural cavity and neutrophil accumulation (B) in the lung, as measured by MPO activity, at 24 hr after ZAP injection was inhibited by 5-AIQ. Each value is the mean ± SEM for $n = 10$ experiments, (★) $P < 0.01$ vs. Sham; (○) $P < 0.01$ vs. ZAP.

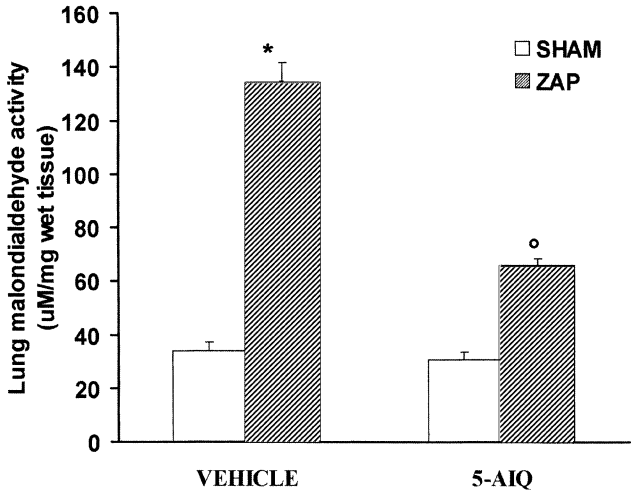

Fig. 3. Effect of 5-AIQ, on MDA levels in the lung. Within 24 hr, pleural injection of ZAP led to an increase in lipid peroxidation of lung tissue (as measured by MDA). 5-AIQ inhibited the peroxidation. Each value is the mean ± SEM for $n = 10$ experiments, (⋆) $P < 0.01$ vs. Sham; (○) $P < 0.01$ vs. ZAP.

bronchial epithelium from ZAP-treated mice. 5-AIQ reduced the degree of PARP activation (Fig. 6C). To verify the binding specificity for PARP, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations, no positive staining was found in the sections indicating that the immunoreaction was positive in all the experiments carried out.

3.4. Effects of 5-AIQ on the release of cytokines

At 24 hr after the injection of ZAP, an increase in the levels of TNFα and IL-1β was observed in pleural exudates when compared to controls (Fig. 7). 5-AIQ attenuated the release of TNFα and IL-1β (Fig. 7).

3.5. 5-AIQ inhibits expression of P-selectin and ICAM-1 in human endothelial cells

From the *in vivo* experiments, the hypothesis emerges that PARP activation is a crucial determinant of the changes of adhesive capability of endothelium. However, the *in vivo* experiments cannot prove a direct correlation between PARP activation and cellular surface expression of adhesion molecules. Therefore, in additional *in vitro* experiments, we sought to investigate whether inhibition of PARS may directly affect endothelial expression of adhesion molecules. Incubation of HUVECs with peroxynitrite or immunostimulation with TNFα (Fig. 8) induced the up-regulation expression of P-selectin and of ICAM-1. Pre-treatment of these cells with 5-AIQ caused a concentration-dependent attenuation of the expression of P-selectin and upregulation of ICAM-1 caused by peroxynitrite or TNFα (Fig. 8).

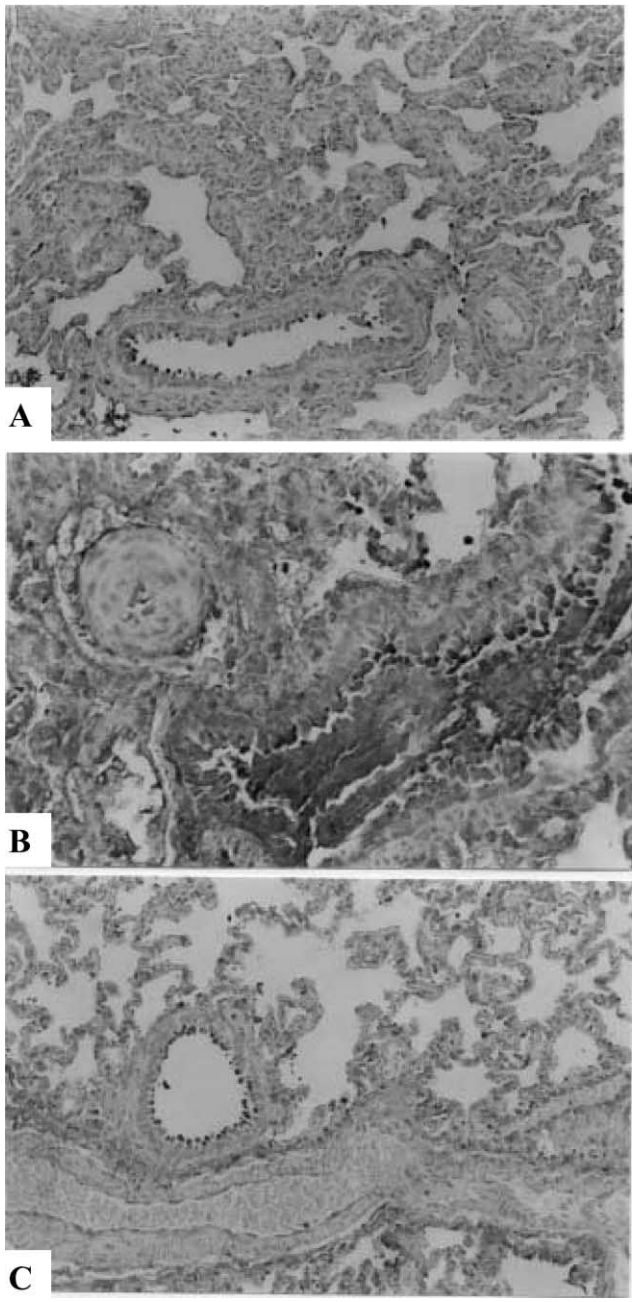

Fig. 4. Immunohistochemical localization of ICAM-1 in the lung. Staining of lung tissue sections obtained from Sham-treated mice with anti-ICAM-1 antibody showed a specific staining along bronchial epithelium, demonstrating that ICAM-1 is constitutively expressed (A). Section obtained from ZAP-treated mice showed intense positive staining for ICAM-1 (B) on bronchial epithelium. The degree of bronchial epithelium staining for ICAM-1 (C) was markedly reduced in tissue section obtained from 5-AIQ-treated mice. Original magnification: 100×. Representative of at least three experiments performed on different experimental days.

3.6. Effects of 5-AIQ on the impairment in cell viability and NAD levels caused by peroxynitrite in human endothelial cells

Exposure of endothelial cells to peroxynitrite for 30 min caused a substantial impairment in cell viability (Fig. 9A). The reduction in cell viability caused by peroxynitrite in

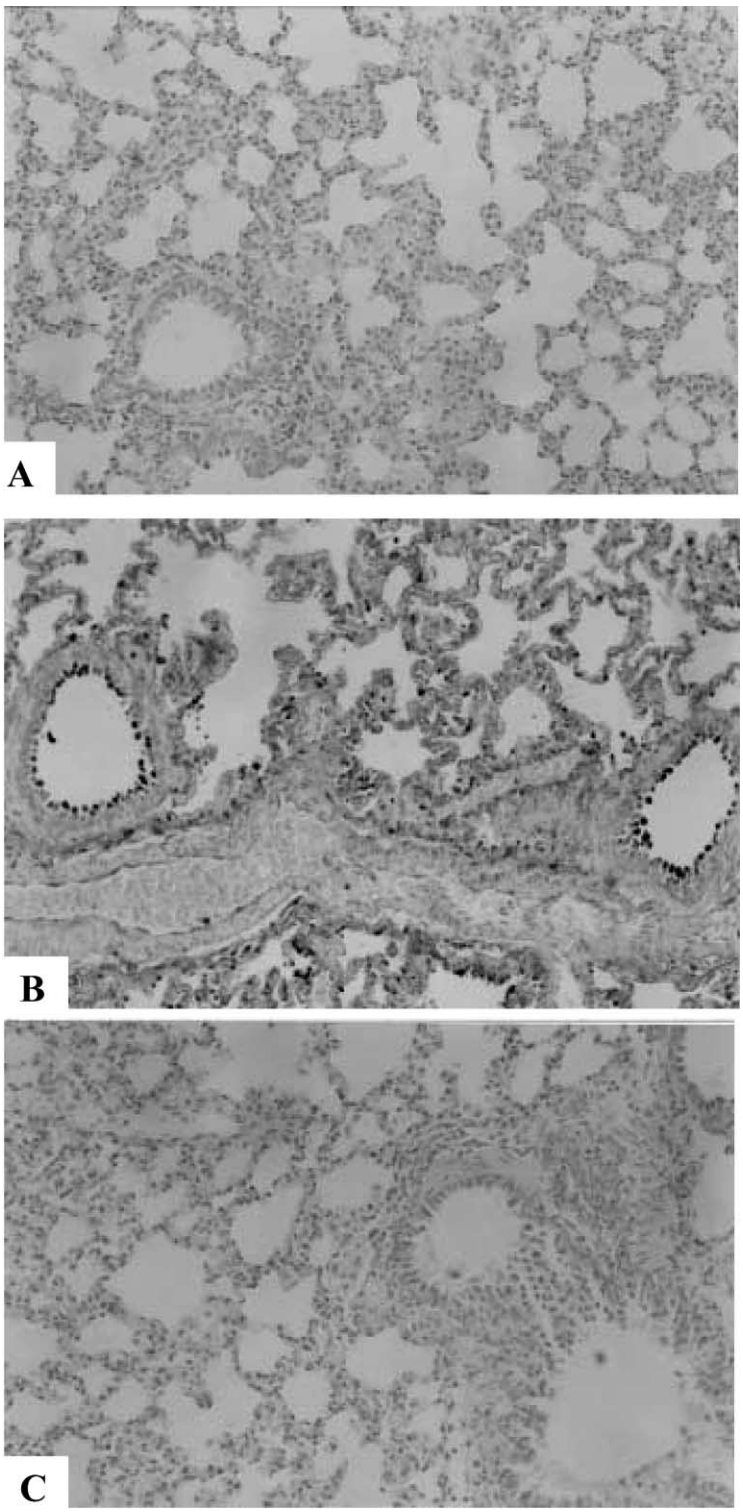

Fig. 5. Immunohistochemical localization of P-selectin in the lung. Lung section from Sham-treated mice revealed no positive staining for P-selectin (A). Section obtained from ZAP-treated mice showed intense positive staining for P-selectin (B) on bronchial epithelium. The degree of bronchial epithelium staining for P-selectin (C) was markedly reduced in tissue section obtained from 5-AIQ-treated mice. Original magnification: 100×. Representative of at least three experiments performed on different experimental days.

these cells was attenuated by 5-AIQ (Fig. 9A). Furthermore, a significant fall in the intracellular levels of NAD^+ (Fig. 9B) was observed in these cells. 5-AIQ caused a concentration-dependent attenuation of the fall in the NAD levels (Fig. 9B).

4. Discussion

Using a new and potent PARS inhibitor (5-AIQ), we demonstrate here that the activation of PARP mediates leukocyte–endothelial interactions by regulating the

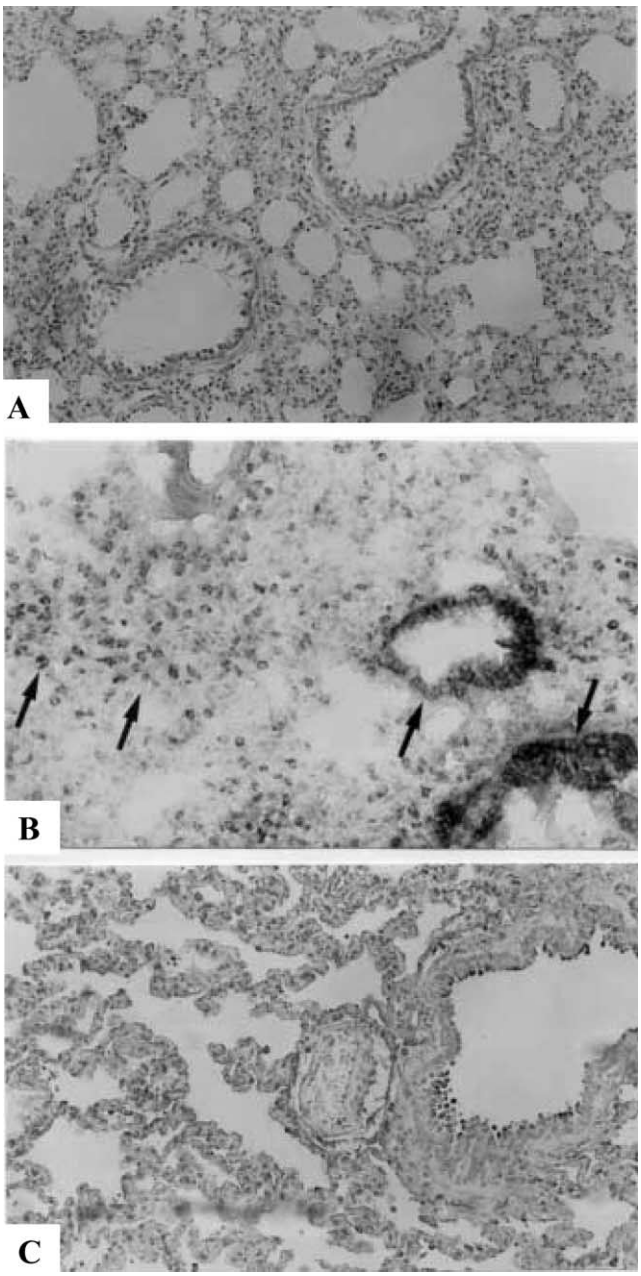

Fig. 6. Immunohistochemical localization of PAR in the lung. No positive staining for PAR (A) was found in the lung section from Sham-administered mice. Immunohistochemistry for PAR (B) show positive staining along the vessels and in the bronchial epithelium from a ZAP-treated mice. The intensity of the positive staining for PARS (C) was significantly reduced in the lung from 5-AIQ-treated mice. Original magnification: ×100. Representative of at least three experiments performed on different experimental days.

expression of P-selectin and ICAM-1 during acute inflammation. In 5-AIQ-treated mice subjected to ZAP-induced pleurisy, the up-regulation of P-selectin and ICAM-1 in the lung was largely attenuated.

Endothelial cells appear to be major regulators of the neutrophil traffic, regulating the process of neutrophil chemoattraction, adhesion and emigration from the vasculature to the tissue. P-selectin is rapidly recruited to the cell surface of either platelets or endothelial cells from preformed storage pools after exposure to, e.g. hydrogen peroxide, trombin, histamine, or complement, and allows the leukocytes to roll along the endothelium [36–40]. ICAM-1 is constitutively expressed on the surface of endothelial cells, is then involved in the neutrophil adhesion [38–40]. Hypoxic or injured endothelial cells synthesize pro-inflammatory cytokines, which can up regulate endothelial expression of the constitutive adhesion molecule ICAM-1 in an autocrine fashion [41,42]. Significant expression of ICAM-1 in microvessels of previously ischemic tissues occurs within 1 hr after reperfusion [43,44]. The up-regulation expression of P-selection and ICAM-1 corresponds with the induction of neutrophil recruitment, which is maximal within the first hour of reperfusion, and persists, at lower rate, in the late phase of reperfusion [45,46]. In accordance with these findings, we observed that ZAP (within 24 hr) induced the appearance of P-selectin on the endothelial vascular wall and up-regulated the surface expression of ICAM-1 on endothelial cells. The PARS inhibitor 5-AIQ abolished the expression of P-selectin and the upregulation of ICAM-1 (Figs. 4C and 5C), but did not affect the constitutive expression of ICAM-1 on endothelial cells (data not shown). These results suggest that inhibition of PARS activity may interfere with the interaction of neutrophils and endothelial cells both at the early rolling phase mediated by P-selectin and at the late firm adhesion phase mediated by ICAM. The absence of an increased expression of the adhesion molecules in the lung tissue of 5-AIQ-treated mice correlated with the reduction of leukocyte infiltration as assessed by the specific granulocyte enzyme myeloperoxidase and with the moderation of the tissue damage as evaluated by histological examination. It is noteworthy, however, that tissue myeloperoxidase activity was not completely abolished. This result is consistent with previous studies demonstrating that constitutive levels of ICAM-1 appear to be sufficient to support a lower degree of CD11/CD18-dependent *trans*-endothelial migration of activated neutrophils [47,48].

Reduction of neutrophil infiltration was also paralleled with the inhibition of PARP immunoreactivity. Our finding (i.e. that PAR staining is reduced in the 5-AIQ animals) coupled with the lung protective effects of PARP inhibition proves the existence of a self-amplifying suicide cycle in which early oxidant production by endothelium activates PARP; the consequent endothelium injury with activation of neutrophil-attractive factors (e.g. ICAM-1 and P-selectin) and neutrophil infiltration leads to further production of oxidants, which ultimately are responsible for the lung injury.

Several *in vitro* and *in vivo* studies have demonstrated that the catalytic activity of the nuclear enzyme PARP, induced by single DNA strand breakage, is a direct result of oxidant injury in conjunction with a variety of immunological stimuli, including bacterial endotoxin and cytokines. More specifically, it has been previously reported

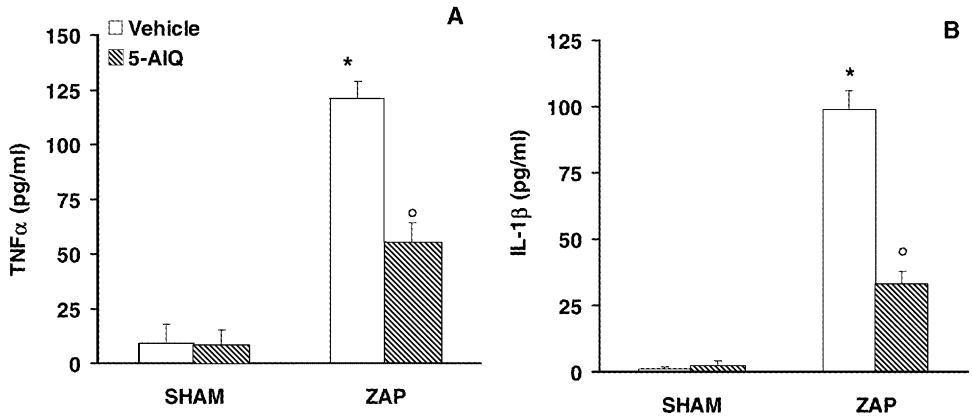

Fig. 7. Pleural injection of ZAP caused at 24 hr an increase in the release of the cytokines TNFα (A), and IL-1β (B). 5-AIQ inhibited TNFα and IL-1β. Each value is the mean ± SEM for $n = 10$ experiments, (★) $P < 0.01$ vs. Sham; (o) $P < 0.01$ vs. ZAP.

that oxidant injury by nitric oxide (NO), peroxynitrite, and/or hydrogen peroxide induces metabolic changes and cytotoxicity in association with the intracellular elevation of PARP activity in macrophages and in pulmonary epithelial, smooth muscle, and endothelial cells [49]. In the present *in vitro* experiments with HUVEC cells, we have demonstrated that oxidant injury by peroxynitrite or TNFα stimulation induces an up-regulation of P-selectin and ICAM-I surface expression, a process that is prevented by the PARP inhibitor 5-AIQ. It is possible that an enhanced adhesion molecule expression may be mediated by an increase in intracellular PARP activity. In conclusion, the data presented here demonstrate that PARP is involved in the regulation of the expression of adhesion molecules and that, consequently, that PARP plays a role in the tissue infiltration of neutrophils. The mode of action of PARP inhibition, as it relates to inhibition of neutrophil infiltration, in the present model is different from the mode of action of PARP inhibitors in the mesenteric microcirculation inflamed with zymosan: in the latter model, PARP appeared to modulate a post-adhesion phenomenon [33]. Taken together, the data presented in the present study and in another recent report [50] demonstrate that PARP regulates the infiltration of neutrophils into the inflamed tissues via a number of distinct mechanisms. The discovery of the concept that PARP regulates neutrophil trafficking may provide new insights in the interpretation of recent reports demonstrating the protective effect of PARS inhibition in experimental models of shock, ischaemia–reperfusion injury and inflammation.

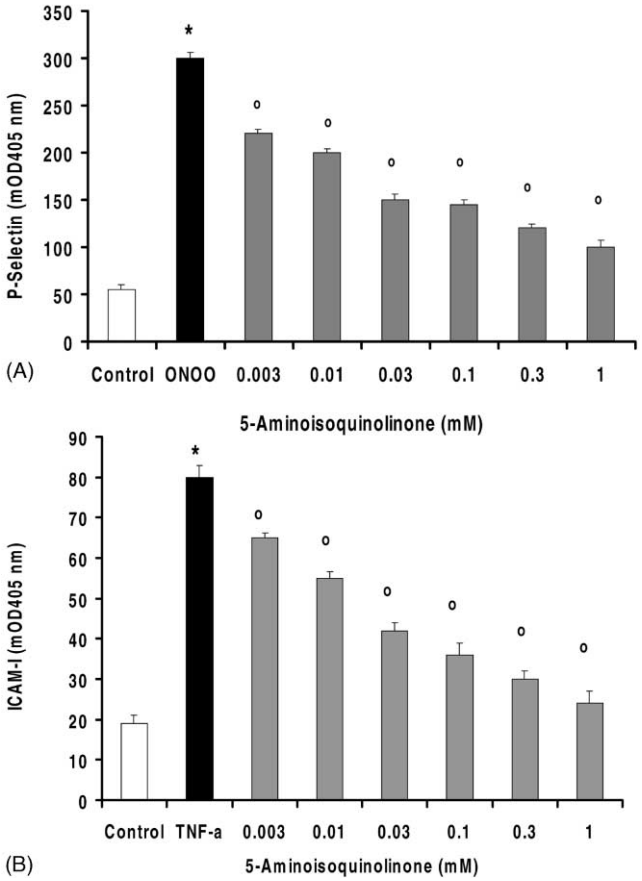

Fig. 8. Effect of 5-AIQ (0.003–10 mM) on expression of P-selectin (A) and ICAM-1 (B) in HUVECs. Expression of P-selectin was induced by incubation of cells with peroxynitrite (10 μmol/L). ICAM-1 was induced by incubation of cells with TNFα (100 unit/mL). On the other hand, 5-AIQ caused a concentration-dependent reduction of the expression of P-selectin and ICAM-1. Each value is the mean ± SEM for 9–12 wells, (★) $P < 0.01$ when compared to control HUVECs; (o) $P < 0.01$ represents a significant inhibitory effect of 5-AIQ.

For instance, there is good evidence that less potent inhibitors of PARP activity (including 3-AB: 10 mg/kg; nicotinamide: 10 mg/kg and 1,5-dihydroxyisoquinoline: 3 mg/kg) reduce by ∼30–50% the degree of tissue injury associated with regional myocardial ischaemia and reperfusion of the heart [26,50,51], the brain [52], the gut [53] and the kidney [54]. Interestingly, a much larger reduction in cerebral infarct size (∼80%) can be detected in mice in which the gene for PARP has been has been disrupted by gene-targeting (PARP knock out or −/− mice) [52]. Thus, it is possible that a much larger therapeutic benefit in conditions associated with inflammation, can be obtained with more potent, water-soluble inhibitors of PARP activity.

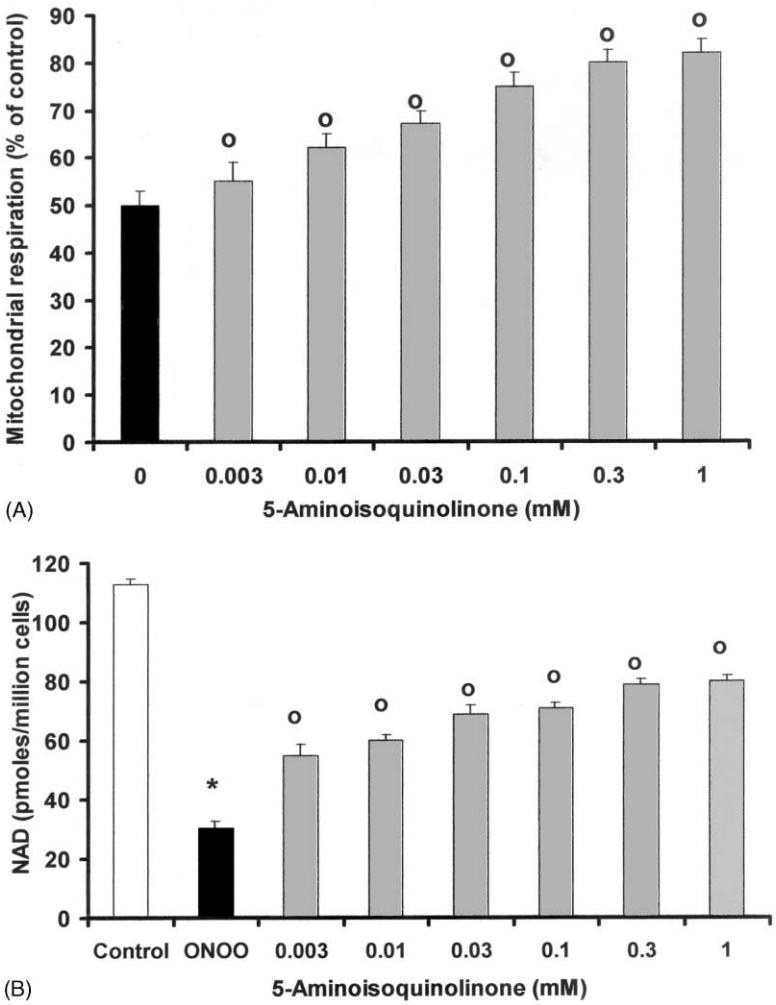

Fig. 9. Effects of PARS inhibition by 5-AIQ (0.003–10 mM) on the cell viability (A) and cellular levels of NAD^+ (B) triggered by authentic peroxynitrite (100 mmol/L) in HUVECs. 5-AIQ prevent in a concentration-dependent manner the suppression of the cell viability and the fall of NAD^+ levels elicited by peroxynitrite, (★) $P < 0.01$ represent when compared to unstimulated controls; (o) $P < 0.05$ represents significant protection by 5-AIQ. Data are expressed as mean ± SEM of $n = 9$–12 wells.

Thus, we propose the following positive feedback cycle in acute lung inflammation: early ROS production ⩾ PARP-related endothelial injury ⩾ PMN infiltration ⩾ more ROS production. Inhibition of PARP would intercept this cycle at the level of endothelial injury. Therefore, to its effect on preserving the cellular energetic status and protecting against oxidant-induced cell necrosis, regulation of neutrophil recruitment may represent a novel important additional anti-inflammatory mode of action of this novel inhibitor of PARP activity.

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References

[1] Vane J, Botting R. Inflammation and the mechanism of action of anti-inflammatory drugs. FASEB J 1987;1:89–96.
[2] Willoughby DA. Cyclo-oxygenase and nitric oxide isoforms in rat carrageenin-induced pleurisy. Br J Pharmacol 1994;113:693–8.
[3] Lefer AM, Lefer DJ. Pharmacology of the endothelium in ischemia–reperfusion and circulatory shock. Ann Rev Pharmacol Toxicol 1993;33:71–90.
[4] Ratych RE, Chuknyska RS, Burkley GB. The primary localisation of free radical generation after anoxia/reoxygenation in isolated endothelial cells. Surgery 1987;102:122–31.

[5] Granger DN, Rutili G, McCord JM. Superoxide radicals in feline intestinal ischemia. Gastroenterology 1981;81:22–3.
[6] Johnson KJ, Varani J, Smolen JE. Neutrophil activation and function in health and disease. In: Coffey RG, editor. Granulocyte response to cytokines. New York: Marcel Dekker, 1992. p. 1–46.
[7] Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA. Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. J Cell Biol 1991;115:223–34.
[8] Patel KD, Zimmerman GA, Prescott SM, McEver RP, McIntyre TM. Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. J Cell Biol 1991;112:749–59.
[9] Lorant DE, Topham MK, Whatley RE, McEver RP, McIntyre TM, Prescott SM, Zimmerman GA. Inflammatory roles of P-selectin. J Clin Invest 1993;92:559–70.
[10] Butcher EC. Specificity of leukocyte–endothelial interactions and diapedesis: physiologic and therapeutic implications of an active decision process. Res Immunol 1993;144:695–8.
[11] Wetheimer SJ, Myers CL, Wallace RW, Parks TP. Intercellular adhesion molecule-1 gene expression in human endothelial cells. J Biol Chem 1999;267:12030–5.
[12] Ueda K, Hayaishi O. ADP-ribosylation. Ann Rev Biochem 1992;54:73–100.
[13] Berger NA. Poly(ADP-ribose) in the cellular response to DNA damage. Radic Res 1985;101:4–15.
[14] Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RG, Cochrane CG. DNA strand breaks activate poly adenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. J Clin Invest 1986;77:1312–20.
[15] Hyslop PA, Hinshaw DB, Halsey WA, Schraufstatter IU, Sauerheber RD, Spragg RG, Jackson JH, Cochrane CG. Mechanisms of oxidant-mediated cell injury: the glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. J Biol Chem 1988;263:1665–75.
[16] Thies RL, Autor AP. Reactive oxygen injury to cultured pulmonary artery endothelial cells: mediation by poly(ADP-ribose) polymerase activation causing NAD depletion and altered energy balance. Arch Biochem Biophys 1991;286:353–63.
[17] Oh-ishi S, Hayashi I, Hayashi M, Yamaki K, Utsunomiya I. Pharmacological demonstration of inflammatory mediators using experimental inflammatory models: rat pleurisy induced by carrageenin and phorbol myristate acetate. Dermatologica 1989;179: 68–71.
[18] Peskar BM, Trautmann M, Nowak P, Peskar BA. 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 1991;33:240–6.
[19] Cuzzocrea S, Costantino G, Mazzon E, Caputi AP. Beneficial effects of raxofelast (IRFI 016), a new hydrophilic Vitamin E-like antioxidant, in carrageenan-induced pleurisy. Br J Pharmacol 1999;126:407–14.
[20] Cuzzocrea S, Zingarelli B, Costantino G, Caputi AP. Beneficial effects of Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), a superoxide dismutase mimetic, in carrageenan-pleurisy. Free Radic Biol Med 1999;26:26–33.
[21] Da Motta JI, Cunha FQ, Vargaftig BB, Ferreira SH. Drug modulation of antigen-induced paw oedema in guinea-pigs: effects of lipopolysaccharide, tumour necrosis factor and leucocyte depletion. Br J Pharmacol 1994;112:111–6.
[22] Carson DA, Set S, Wasson B, Carrera CJ. DNA strand breaks, NAD metabolism and programmed cell death. Exp Cell Res 1986;164:273–81.
[23] Cuzzocrea S, Zingarelli B, Gilard E, Hake P, Salzman AL, Szabó C. Protective effects of 3-aminobenzamide, an inhibitor of poly(ADP-ribose) synthase in carrageenan-induced models of local inflammation. Eur J Pharmacol 1998;342:67–76.
[24] Szabó C, Viràg L, Cuzzocrea S, Scott GS, Hake P, O'Connor M, Zingarelli B, Ma Y, Hirsch R, Boiovin GP, Salzman AL, Kun E. Protection against peroxynitrite-induced fibroblast injury and arthritis development by inhibition of poly(ADP-Ribose) synthetase. Proc Natl Acad Sci USA 1998;95:3867–72.
[25] Bowes J, Mcdonald MC, Piper J, Thiemermann C. Inhibitors of poly(ADP-ribose) synthetase protect rat cardiomyocytes against oxidant stress. Cardiovasc Res 1999;41:126–34.
[26] Bowes J, Piper J, Thiemermann C. Inhibitors of the activity of poly(ADP-ribose) synthetase reduce the cell death caused by hydrogen peroxide in human cardiac myoblasts. Br J Pharmacol 1998;124:1760–6.
[27] Mc Donald MC, Mota-Filipe H, Wright JA, Abdelrahman M, Threadgill MD, Thompson A, Thiemermann C. Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly(ADP-ribose) polymerase on the organ injury and dysfunction caused by haemorrhagic shock. Br J Pharmacol 2000;130:843–50.
[28] Yarwood H, Nourshargh S, Brain S, Williams TJ. Effect of dexamethasone on neutrophil accumulation and oedema formation in rabbit skin: an investigation of site of action. Br J Pharmacol 1993;108:959–66.
[29] Henriques MG, Rae GA, Cordeiro RS, Williams TJ. Endothelin-1 inhibits PAF-induced paw oedema and pleurisy in the mouse. Br J Pharmacol 1992;106:579–82.
[30] Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Clin Invest Dermatol 1982;78:206–9.
[31] Laight DW, Lad N, Woodward B, Waterfall JF. Assessment of myeloperoxidase activity in renal tissue after ischemia/reperfusion. Eur J Pharmacol 1994;292:81–8.
[32] Ohkawa H, Ohishi H, Yagi K. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. Anal Biochem 1979;95:351–8.
[33] Szabo C, Lim LHK, Cuzzocrea S, Getting SJ, Zingarelli B, Flower RJ, Salzman AL, Perretti M. Inhibition of poly(ADP-ribose) synthetase exerts anti-inflammatory effects and inhibits neutrophil recruitment. J Exp Med 1997;186:1041–9.
[34] Cuzzocrea S, Caputi AP, Zingarelli B. Peroxynitrite-mediated DNA strand breakage activates poly(ADP-ribose) synthetase and causes cellular energy depletion in carrageenan-induced pleurisy. Immunology 1998;93:96–101.
[35] Zingarelli B, O'Connor M, Wong H, Salzman AL, Szabó C. Peroxynitrite-mediated DNA strand breakage activates poly-adenosine diphosphate ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipolysaccharide. J Immunol 1996;156:350–8.
[36] Geng JG, Bevilacqua MP, Moore KL, McIntyre TM, Prescott SM, Kim JM, Bliss GA, Zimmerman, McEver RP. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature 1990;343:757–60.
[37] Lawrence MB, Spinger TA. Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell 1991;65:859–73.
[38] Mulligan MS, Polley MJ, Bayer RJ, Nunn MF, Paulson JC, Ward PA. Neutrophil-dependent acute lung injury: requirement for P-selectin (GMP-140). J Clin Invest 1992;90:1600–7.
[39] Davenpeck KL, Gauthier TW, Albertine KH, Lefer AM. Role of P-selectin in microvascular leukocyte–endothelial interaction in splanchnic ischemia–reperfusion. Am J Physiol 1994;267:H622–30.
[40] Farhood A, McGuire GM, Manning AM, Miyasaka M, Smith CW, Jaeschke H. Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia–reperfusion injury in rat liver. J Leukoc Biol 1995;57:368–74.
[41] Shreeniwas R, Koga S, Karakurum M, Pinsky D, Kaiser E, Brett J, Wolitzky BA, Norton C, Plocinski J, Benjamin W, Kurns DK, Goldestein A, Stern D. Hypoxia-mediated induction of endothelial cell interleukin-1 alpha: an autocrine mechanism promoting expres-

sion of leukocyte adhesion molecules on the vessel surface. J Clin Invest 1992;90:2333–9.

[42] Luscinskas FW, Ding H, Lichtman AH. P-selectin and vascular cell adhesion molecule 1 mediate rolling and arrest, respectively, of CD4+ T lymphocytes on tumor necrosis factor alpha-activated vascular endothelium under flow. J Exp Med 1995;181:1179–86.

[43] Clark WM, Lauten JD, Lessov N, Woodward W, Coull BM. Time course of ICAM-1 expression and leukocyte subset infiltration in rat forebrain ischemia. Mol Chem Neuropathol 1995;26:213–30.

[44] Horie Y, Wolf R, Iyasaka M, Anderson DC, Granger DN. Leukocyte adhesion and hepatic microvascular responses to intestinal ischemia/reperfusion in rats. Gastroenterology 1996;111:666–73.

[45] Gauthier TW, Davenpeck KL, Lefer AM. Nitric oxide attenuates leukocyte–endothelial interaction via P-selectin in splanchnic ischemia–reperfusion. Am J Physiol 1994;267:G562–8.

[46] Hawkins HK, Entman ML, Zhu JY, Youker KA, Berens K, Dore M, Smith CW. Acute inflammatory reaction after myocardial ischemic injury and reperfusion: development and use of a neutrophil-specific antibody. Am J Pathol 1996;148:1957–69.

[47] Furie MB, Tancinco MC, Smith CW. Monoclonal antibodies to leukocyte integrins CD11a/CD18 and CD11b/CD18 or intercellular adhesion molecule-1 inhibit chemoattractant-stimulated neutrophil transendothelial migration *in vitro*. Blood 1991;78:2089–97.

[48] Kukielka GL, Youker KA, Hawkins HK, Perrard JL, Michael LH, Ballantyne CM, Smith CW, Entman ML. Regulation of ICAM-1 and IL-6 in myocardial ischemia: effect of reperfusion. Ann New York Acad Sci 1994;723:258–70.

[49] Szabó C, Dawson VL. Role of poly(ADP-ribose) synthetase in inflammation and ischaemia–reperfusion. Trends Pharmacol Sci 1998;19:287–98.

[50] Zingarelli B, Salzman AL, Szabo C. Genetic disruption of poly(ADP-ribose) synthetase inhibits the expression of P-selectin and intercellular adhesion molecule-1 in myocardial ischemia/reperfusion injury. Circ Res 1998;83:85–94.

[51] Thiemermann C, Bowes J, Myint F, Vane JR. Inhibition of the activity of poly(ADP-ribose) synthetase reduces ischemia-reperfusion injury in the heart and skeletal muscle. Proc Natl Acad Sci USA 1997;94:679–83.

[52] Eliasson MJL, Sampei K, Mandir AS, Hurn PD, Traystman RJ, Bao J, Pieper A, Wang Z, Dawson TM, Snyder SH, Dawson VL. Poly (ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischaemia. Nature Med 1997;3:1089–95.

[53] Cuzzocrea S, Zingarelli B, Costantino G, Szabo A, Salzman AL, Caputi AP, Szabo C. 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 1997;121:1065–74.

[54] Chatterjee PK, Cuzzocrea S, Thiemermann C. Inhibitors of poly(ADP-ribose) synthetase protect rat proximal tubular cells against hydrogen peroxide-mediated oxidant stress. Kidney Int 1999;56:973–84.