Inhibition of poly (ADP-ribose) Synthetase Attenuates Neutrophil Recruitment and Exerts Antiinflammatory Effects

By Csaba Szabó,* Lina H.K. Lim,† Salvatore Cuzzocrea,* Stephen J. Getting,‡ Basilia Zingarelli,* Roderick J. Flower,‡ Andrew L. Salzman,* and Mauro Perretti‡

From the *Children’s Hospital Medical Center, Division of Critical Care, Cincinnati, Ohio 45229; and ‡Department of Biochemical Pharmacology, The William Harvey Research Institute, London EC1M 6BQ, United Kingdom

Summary

A cytotoxic cycle triggered by DNA single-strand breakage and poly (ADP-ribose) synthetase activation has been shown to contribute to the cellular injury during various forms of oxidant stress in vitro. The aim of this study was to investigate the role of poly (ADP-ribose) synthetase (PARS) in the process of neutrophil recruitment and in development of local and systemic inflammation. In pharmacological studies, PARS was inhibited by 3-aminobenzamide (10–20 mg/kg) in rats and mice. In other sets of studies, inflammatory responses in PARS−/− mice were compared with the responses in corresponding wild-type controls. Inhibition of PARS reduced neutrophil recruitment and reduced the extent of edema in zymosan- and carrageenan-triggered models of local inflammation. Moreover, inhibition of PARS prevented neutrophil recruitment, and reduced organ injury in rodent models of inflammation and multiple organ failure elicited by intraperitoneal injection of zymosan. Inhibition of PARS also reduced the extent of neutrophil emigration across murine mesenteric postcapillary venules. This reduction was due to an increased rate of adherent neutrophil detachment from the endothelium, promoting their reentry into the circulation. Taken together, our results demonstrate that PARS inhibition reduces local and systemic inflammation. Part of the antiinflammatory effects of PARS inhibition is due to reduced neutrophil recruitment, which may be related to maintained endothelial integrity.

In vitro studies have demonstrated that oxidative injury in various cell types is related in part to DNA single-strand breakage and the consequent activation of the nuclear enzyme poly (ADP-ribose) synthetase (PARS). Massive ADP ribosylation of nuclear proteins by PARS then results in cellular energy depletion and injury (1–6).

This present work was designed to elucidate (a) whether inhibition of PARS exerts antiinflammatory effects in various models of local and systemic inflammation, and (b) whether inhibition of PARS affects neutrophil recruitment during inflammation. In our studies we used 3-aminobenzamide, a prototypical pharmacological inhibitor of PARS (1–7), and genetically engineered mice lacking functional PARS enzyme (PARS−/−) (8).

Materials and Methods

Animals. Male Swiss Albino mice (20–22 g; Interfauna, Huntingdon, United Kingdom) were used for investigation of the effect of 3-aminobenzamide in the zymosan peritonitis model, and in leukocyte–endothelium interaction by intravital microscopy.

Male Wistar rats (300–320 g; Charles River Laboratories, Wilmington, MA) were used for investigation of the role of PARS in the pathogenesis of the carrageenan-induced paw edema, and in zymosan-induced multiple organ failure.

Responses in PARS−/− and PARS+/+ mice (8) (129/Sv × C57BL6, 20–22 g, kind gifts of Dr. Z.Q. Wang, Institute of Molecular Pathology, Vienna, Austria) were compared to assess the role of PARS in the pathogenesis of the zymosan-induced multiple organ failure in mice.

Zymosan-induced Peritonitis in Mice Treated with 3-aminobenzamide. Peritonitis was induced by intraperitoneal injection of zymosan (12.5 mg/kg) in 0.5 ml PBS (0.1 M, pH 7.4) (9). At 2 or 4 h, animals were killed by CO2 exposure, and peritoneal cavities were washed with 3 ml of PBS containing 3 mM EDTA. Aliquots of the lavage fluids were then stained with Turk’s solution.

Abbreviations used in this paper: AST, aspartate aminotransferase; LDH, lactate dehydrogenase; MPO, myeloperoxidase; PARS, poly (ADP-ribose) synthetase.

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and differential counting was performed using a hemocytometer and a light microscope. Data are reported as 10^6 PM N per mouse. The large predominance (>98%) of neutrophils in the PM N population in 2- and 4-h lavage fluids was confirmed in cytospin preparations stained with May-Gr"unwald and Giemsa. Lavage fluids were then centrifuged at 400 g for 10 min, and supernatants stored at −20°C before evaluation of β-glucuronidase activity, according to a published protocol (10).

3-Aminobenzamide was dissolved in saline, and was sonicated at appropriate concentrations for injecting 100–150 μl per mouse. In the first set of experiments, vehicle or 3-aminobenzamide (10 or 20 mg/kg) was given intravenously immediately before intraperitoneal administration of zymosan. In another set of experiments, vehicle or the PAR S inhibitor (2, 10, or 20 mg/kg) was given intravenously 2 h after zymosan administration. In control experiments, 3-aminobenzoic acid, a structural analog of 3-aminobenzamide with no inhibitory effect on PAR S (6), was used at a dose of 10 mg/kg.

PM N Adhesion and Migration in Mice Treated with 3-aminobenzamide. Mice were fanned overnight before experimentation. Animals were injected intraperitoneally with 12.5 mg/kg zymosan (in 0.5 ml sterile saline) or saline (controls), and were left at liberty until the beginning of the experiment. 4 h later, mesenteries were prepared as described (11). Mice were anesthetized with di-azepam (60 μl subcutaneously) and Hypnorm\textsuperscript{TM} (30 μl intramuscularly; Janssen Pharmaceutical Ltd., Oxford, United Kingdom). A tracheotomy was performed to facilitate breathing. Cautery incisions were made along the abdominal region, and the mesenteric vascular bed was exteriorized and placed on a plexiglass stage for viewing. The preparation was then mounted on an Axioskop FS (Carl Zeiss, Inc., Welwyn Garden City, United Kingdom) with a water immersion objective lens (×40; Carl Zeiss, Inc.) to observe the microcirculation. The preparation was transilluminated with a 12-V, 100-W halogen light source. Images were displayed and recorded for subsequent offline analysis. Mesenteries were superfused with 37°C bicarbonate-buffered solution (g/liter: NaCl 7.71, KCl 0.25, MgSO\textsubscript{4} 0.14, NaHCO\textsubscript{3} 1.51, and CaCl\textsubscript{2} 0.22, pH 7.4, gassed with 5% CO\textsubscript{2}/95% N\textsubscript{2}). One to three randomly selected postcapillary venules (diameter between 20-40 μm, length of at least 100 μm) were observed for each mouse. Adhesion was monitored by counting, for each vessel, the number of adherent leukocytes in a 100-μm length. Leucocyte emigration from the microcirculation into the tissue was quantified by counting the number of cells that had emigrated out of the vessel wall into the vessel lumen in parallel with the 100-μm vessel segment.

In the first set of experiments, vehicle or 3-aminobenzamide was given intravenously immediately before intraperitoneal administration of zymosan. In a separate set of experiments, mesenteries were exposed 2 h after zymosan injection as described above, and vessels with a congruous number of adherent leukocytes (five to eight per branch) were chosen. Either PBS (100 μl) or 3-aminobenzamide (20 mg/kg) was given intravenously through the tail vein, and the fate of the adherent leukocytes was monitored for 10 min.

Carrageenan-induced Paw Edema in Rats Treated with 3-aminobenzamide. Rats received a subplantar injection 0.1 ml saline containing 1% α-carrageenan into the right hind paw (12). The phlogogenic agent was given together with vehicle, with 3-aminobenzamide, or with the inactive structural analogue 3-aminobenzoic acid (25 μg/paw). The test agents were solubilized in saline solution, and the injection volume was 0.1 ml. Control animals received the same volume of vehicle. The volume of the paw was measured by a plethysmometer (Kent Laboratories, Kent, WA) (12).

M yeloperoxidase (MPO) activity, an index of PM N accumulation, was determined as previously described (13). Paw tissues, collected at the specified time, were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0), and were 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 tetramethylbenzidine (1.6 mM) and 0.1 mM H\textsubscript{2}O\textsubscript{2}. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. M yeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxidase min at 37°C, and was expressed in milliunits per 100 mg wt of wet tissue.

Zymosan-induced Peritonitis and Multiple Organ Failure in Rats and Mice. Rats or mice were injected with 500 mg/kg zymosan i.p. to induce multiple organ failure (14). 18 h after zymosan injection, animals were killed by CO\textsubscript{2} exposure. Plasma samples were taken, and lactate dehydrogenase (LDH) levels and serum aspartate aminotransferase (AST) levels were determined by a clinical laboratory. The abdomen was carefully opened, and the peritoneal cavity was washed with 2 ml of saline solution with heparin (5 U/ml) and indomethacin (10 μg/ml).

Lavage fluids were then collected and measured, and exudate values were obtained after subtracting the volume injected. Differential cell counts were determined as described above. Lung, liver, and small intestinal MPO activities were determined as described for the paw tissues. For histopathological examination, tissues were fixed in 10% neutral-buffered formaldehyde for 5 d, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin.

The above experiments were performed in rats and mice. In rats, responses in vehicle-pretreated animals and responses in animals pretreated with 3-aminobenzamide (10 mg/kg, 10 min before zymosan and every 6 h thereafter) were compared. In mice, responses in PAR S−/− and PAR S+/+ animals were compared.

Data Analysis. All values in the figures and text are expressed as mean ± standard error of the mean of n observations, where n represents the number of animals studied. Data sets were examined by one- and two-way analysis of variance, and individual group means were compared with Student’s unpaired t test. Non-parametric data were analyzed with the Fisher’s exact test. A P value <0.05 was considered significant.

Results

3-Aminobenzamide Reduces the Local Inflammatory Response in the Zymosan Peritonitis Model. Zymosan injection into murine peritoneal cavities produced a time-dependent PM N accumulation that was maximal at 4 h (15). Pretreatment of animals with 3-aminobenzamide (10–20 mg/kg) resulted in a significant reduction of PM N accumulation (Fig. 1 A). The PAR S inhibitor did not modify the extent of cell activation at the inflammatory site, assessed as β-glucuronidase activity in the lavage fluids. For instance, β-glucuronidase activity was 66 ± 8 and 58 ± 17 U per 10^6 PM N in the presence of PBS and 3-aminobenzamide (20 mg/kg) treatment, respectively (n = 6). The inhibitory action of 3-aminobenzamide on PM N elicitation was not the result of an indirect effect on the number of circulating leukocytes.
since 3-aminobenzamide did not significantly alter the profile of circulating blood cells (Table 1). The inactive structural analog of 3-aminobenzamide, 3-aminobenzoic acid, did not alter PMN recruitment (Fig. 1).

Fig. 1 B shows the effect of 3-aminobenzamide treatment (given 2 h after zymosan) on the number of cells recovered from the peritoneal cavities at the 4-h time point. In the period between 2 and 4 h, PMNs accumulated in response to zymosan with a high rate of influx (~3.5 × 10⁶ cells/h), and administration of 3-aminobenzamide was highly effective in reducing cell recruitment. More than 70% reduction in PMN influx was seen with 10 or 20 mg/kg 3-aminobenzamide, whereas the PAR S inhibitor was inactive at 2 mg/kg (Fig. 1 B).

3-Aminobenzamide Inhibits PMN Emigration Across Mice Mesenteric Postcapillary Venules Challenged with Zymosan. The effect of 3-aminobenzamide on leukocyte-endothelial interaction was monitored by intravital microscopy. Zymosan injection induced high numbers of adherent and emigrated leukocytes in mouse mesenteric postcapillary venules 4 h after administration (Fig. 2). Treatment of mice with 3-aminobenzamide (20 mg/kg) immediately before zymosan did not modify the extent of cell adhesion (Fig. 2 A), but significantly reduced the number of cells that emigrated outside the postcapillary venules (Fig. 2 B).

We then investigated the fate of the adherent leukocytes in the presence or absence of pharmacological inhibition of PAR S in the mesenteries inflamed with zymosan. Postcapillary venules were visualized 2 h after zymosan injection. Subsequently, vehicle or 3-aminobenzamide (20 mg/kg) was given intravenously. While most of the adherent cells emigrated through the endothelium of the postcapillary venules in the vehicle-treated animals, a large majority detached from the endothelial surface after treatment with the PAR S inhibitor (Fig. 3 A). In vehicle treated mice, only 25% of adherent cells detached after vehicle injection, whereas most leukocytes emigrated (Fig. 3 B). In contrast, in response to treatment with 3-aminobenzamide, cells continued to detach and reenter the circulation. A total of 80% of adherent cells detached after administration of the PAR S inhibitor (Fig. 3 B).

Although 3-aminobenzamide did not apparently modify zymosan-induced cell adhesion at a fixed time point, it increased the rate of cell detachment from the postcapillary venule endothelium. In this protocol, only the fate of cells that were adherent before 3-aminobenzamide or vehicle intravenous challenge was followed, without considering newly adhered cells. The two processes (adhesion and detachment) equilibrated themselves, explaining why no difference in the degree of cell adhesion was seen at a single time point. Nonetheless, following the fate of adherent cells immediately after 3-aminobenzamide administration permitted identification of the process that was selectively

Table 1. Lack of Effect of 3-aminobenzamide on Circulating Peripheral Blood Leukocyte Numbers

| Treatment          | PMN       | Monocytes | Lymphocytes |
|--------------------|-----------|-----------|-------------|
|                    | 10⁶/ml    | %         | 10⁶/ml      | %         | 10⁶/ml      | %         |
| PBS                | 0.86 ± 0.21 (10.4) | 1.00 ± 0.21 (13.2) | 5.90 ± 0.87 (76.4) |
| 3-aminobenzamide   | 0.92 ± 0.06 (14.2) | 1.03 ± 0.24 (15.3) | 4.82 ± 0.59 (70.5) |

Mice received either PBS (100 µl i.v.) or 3-aminobenzamide (20 mg/kg i.v.) 4 h before blood collection by cardiac puncture. Differential leukocyte counting was performed after staining in Turk’s solution. Data are mean ± SEM of six mice per group.
affected by the drug. The net result is a diminished cell emigration across postcapillary venules and reduced PMN tissue infiltration.

3-Aminobenzamide Reduces Carrageenan-induced Rat Paw Edema. Based on the marked effects of 3-aminobenzamide on neutrophil recruitment in the zymosan-induced models of inflammation, we investigated the potential antiinflammatory effect of this PARS inhibitor in experimental models of inflammation where neutrophil recruitment plays a crucial role. One of these processes is the carrageenan-induced local inflammation and tissue injury (16, 17). In the carrageenan-induced rat paw edema model, pretreatment of the animals with 3-aminobenzamide (25 μg/paw) caused a marked reduction in edema development (Fig. 4A), and significantly reduced the increase in tissue levels of myeloperoxidase, indicative of reduced neutrophil accumulation in the paw (Fig. 4B). The degree of inhibition by 3-aminobenzamide of the paw edema was less pronounced (<50% inhibition) at 1–2 h, but it was more pronounced at 4 h (>75% inhibition). This difference in the degree of inhibition may be related to the nature of the various mediators involved in the pathogenesis of carrageenan-induced paw edema. In the early stage, endogenous amines and prostaglandins play an important role, while infiltrating PMNs play a crucial role in the more delayed injury (3–4 h) (12, 16–18). Nevertheless, peroxynitrite production, formed by the reaction of superoxide with NO (the latter produced by constitutive NO synthase isoforms), is well established, even in the early phase of inflammation in this model (19, 20). Similar to the results of the zymosan peritonitis studies, the inactive structural analog of 3-aminobenzamide, 3-aminobenzoic acid, did not alter the development of paw edema (n = 5, data not shown).

3-Aminobenzamide and PAR$^S{\text{−/}}$ Phenotype Reduce the Systemic Inflammation (Multiple Organ Failure) Induced by Zymosan. Intraperitoneal injection of a high dose of zymosan induces nonseptic shock and multiple organ failure, with intensive PMN migration into various organs (14, 21–23). In our experiments we chose the 18-h time point, and observed strong peritoneal inflammation (detected as exudation and leukocyte accumulation) and increased PMN infiltration into various organs (Fig. 5). In the wild-type (PAR$^S{\text{+/}}$) control mice, zymosan injection increased se-
rum AST and LDH levels to 170 ± 25 and 137 ± 24% of control, respectively (P < 0.05; n = 5). Treatment of rats with 3-aminobenzamide (10 mg/kg), or the absence of PAR S in mice (animals with the PAR S<sup>−/−</sup> genotype) resulted in a pronounced reduction of exudate volumes and leukocyte counts in the peritoneum, and significantly attenuated the zymosan-induced increase in the MPO activity in the organs studied (Fig. 5). There were no significant increases in serum AST and LDH levels in the PAR S<sup>−/−</sup> mice in response to zymosan injection (respective values were 72 ± 20% and 97 ± 32% of control; n = 5). There was also a marked reduction in the degree of histological injury in the PAR S<sup>−/−</sup> mice after zymosan injection when compared with the response to zymosan in the PAR S<sup>+/+</sup> mice. The extent of zymosan-induced mononuclear cell infiltration and the degree of the histological damage were markedly reduced in the lung (Fig. 6) and liver (not shown) of the PAR S<sup>−/−</sup> mice when compared with the wild-type PAR S<sup>+/+</sup> control mice.

**Discussion**

PAR S Inhibition Reduces PMN Accumulation in Inflammation. The main finding of this study is that PAR S inhibition (by a pharmacological approach or by the use of genetically engineered animals) reduces PMN recruitment and accumulation into inflammatory tissue sites. Extravasated PMNs become activated once in the inflammatory sites, secreting a variety of substances such as growth factors, chemokines and cytokines, complement components, proteases, NO, reactive oxygen metabolites, and peroxynitrite, all important mediators of tissue injury (24–26). Pre-
vention of neutrophil-dependent inflammatory pathways is likely to contribute to the reduced fluid extravasation and improved histologic status after PARS inhibition. There are recent reports from our laboratory and from other groups showing the protective effect of PARS inhibitors in experimental models of stroke (4, 27), endotoxic shock (28), and ischemia-reperfusion injury (29). Because extensive activation of PARS due to massive oxidant-mediated DNA injury can lead to pronounced NAD$^+$ and ATP depletion in various tissues, it was generally assumed that the mode of protection by inhibitors of PARS is directly related to improved metabolic status of the target tissues in these models (1–6, 27, 28). In fact, in in vitro studies, our group and other investigators have observed that hydrogen peroxide, oxyradical- or peroxynitrite-induced cellular injury is ameliorated by pharmacological inhibition of PARS (1–6, 27, 28), or in cells derived from the PARS$^{-/-}$ mice, when compared to corresponding wild-type controls (6, 30). Based on the data presented in this study, however, we propose that reduced neutrophil recruitment represents an important additional mechanism for the antiinflammatory effects provided by PARS inhibition.

Although PARS inhibition was effective in reducing PMN recruitment in all inflammatory models tested, paradoxically, the protective effects appeared to be more pronounced in more severe forms and more delayed stages of inflammation (compare, for example, the effects of 3-aminobenzamide on the delayed versus the early phase of zymosan peritonitis (Fig. 1) or carrageenan paw edema (Fig. 4). This effect may be related to the fact that PARS activation and related cellular alterations mainly occur under conditions of more severe oxidant stress.

It is likely that inhibition of PARS exerts antiinflammatory effects both independently of PMN accumulation and via inhibition of PMN influx into the tissues. An example for a PMN-independent protection is inhibition of paw edema development in the early phase of carrageenan edema; 1–2 h after carrageenan injection, only a small degree of PMN infiltration into the paw tissue can be detected (16–20). Such early protection by PARS inhibitors may be related to protection against early, PMN-independent oxidant injury. A likely species responsible for such effects is peroxynitrite, an oxidant produced by the reaction of NO and superoxide anion in this early stage of inflammation (19, 20). There is
now good evidence that many cytotoxic effects of peroxynitrite (suppression of cellular metabolism, epithelial hyperpermeability, endothelial dysfunction) are at least in part due to PARS activation (1–6, 28, 31).

The antineutrophil and antiinflammatory effects of 3-aminobenzamide were likely to be related to PARS inhibition, rather than some other pharmacological action of this agent, since (a) the inactive analog of 3-aminobenzamide (3-aminobenzoic acid) did not affect neutrophil recruitment or edema formation, and (b) the antiinflammatory effects of 3-aminobenzamide were also reproducible in the PARS−/− animals in the zymosan model of multiple organ failure. With regards to 3-aminobenzoic acid, it is noteworthy that in contrast to 3-aminobenzamide, 3-aminobenzoic acid does not protect endothelial cells against oxidant injury (6), or tissues against reperfusion injury (29).

PARS Modulates a Postadhesion Phenomenon. What, then, is the mechanism of protection against PMN recruitment provided by PARS inhibition? The data presented in this study provide evidence that the effects of PARS inhibition are mainly due to interference with PMN postadhesion phenomena. The strongest indication for this conclusion derives from our experiments using intravital microscopy, which allows characterization of temporally related processes such as rolling, adhesion, and emigration (32–35). In our model, zymosan challenge produced a significant degree of cell adhesion and emigration in the mouse mesenteric microcirculation. Treatment of mice with 3-aminobenzamide did not modify zymosan-induced cell adhesion to any extent, but the drug suppressed the degree of cell emigration. This result clearly indicated that 3-aminobenzamide was affecting postadhesion phenomena such that only the number of emigrated cells was altered when analysis was done at a fixed time point (4 h). To further investigate this phenomenon, an appropriate protocol was set up to monitor the adherent leukocytes in real time. Inflammation in the mouse mesentery was induced by zymosan, and postcapillary venules with a congruous number of adherent cells were selected, such that their fate could be monitored after intravenous challenge with 3-aminobenzamide or vehicle. Under these conditions, the PARS inhibitor produced a marked phenomenon of detachment. A similar, higher incidence of leukocyte detachment has been described in response to dexamethasone (33).

The mechanisms regulating leukocyte emigration through the gap formed between adjacent endothelial cells in inflammatory conditions are incompletely understood. In this respect, adhesion molecules such as leukocyte integrins, as well as endothelial intercellular adhesion molecule-1 and platelet-endothelial cell adhesion molecule-1, have been shown to play an important role (32, 34). Since PARS regulates the expression of various genes (36–38), the possibility that PARS may alter the expression of adhesion receptors involved in postadhesion/emigration processes may be proposed. The immediate course (within minutes) of the leukocyte detachment seen after 3-aminobenzamide administration, however, would argue against a mechanism related to altered expression of adhesion molecules, at least in this experimental setting. Endothelial-derived NO inhibits PMN infiltration into the inflammatory tissue sites. In this respect, there are a number of reports demonstrating that oxidant injury to the vascular endothelium, triggered by oxyradicals or by peroxynitrite, is in part mediated by PARS activation (6, 39). Inhibition of PARS has been shown to improve the morphology, metabolic status, and function of the vascular endothelium under oxidant stress (6, 39). Thus, PARS may modulate PMN emigration by altering the metabolic/functional status of the vascular endothelium.

These data, coupled with a number of recent observations, suggest that PARS activation plays a role in oxidant injury in various forms of inflammation and reperfusion injury. These data emphasize the importance of neutrophil recruitment blockade for the protection provided by PARS inhibition. This effect, coupled with a direct cytoprotective effect of PARS inhibition against oxidant injury (1–6, 28, 30, 39), may explain the antiinflammatory effects seen with PARS inhibition. Based on these data, we propose that pharmacological inhibition of PARS represents a novel strategy for antiinflammatory therapy.

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Address correspondence to Dr. Csaba Szabó, Children's Hospital Medical Center, Division of Critical Care, 3333 Burnet Avenue, Cincinnati, OH 45229. Phone: 513-636-8714; Fax: 513-636-4892.

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