Role of Peroxynitrite as a Mediator of Pathophysiological Alterations in Experimental Pneumococcal Meningitis

S. Kastenbauer, U. Koedel, and H. W. Pfister

Department of Neurology, Grosshadern Clinic, Ludwig-Maximilians-University, Munich, Germany

This study investigated the role of peroxynitrite in an adult rat model of pneumococcal meningitis. Immunohistochemically, nitrotyrosine residues, as a marker for peroxynitrite formation, were detected perivascularly and in proximity to inflammatory cells in the subarachnoid space. Nitrotyrosine immunoreactivity was colocalized with blood-brain barrier breach, which was visualized by fluorescence microscopy after intravenous application of Evans blue. Treatment of infected rats with uric acid (300 mg/kg intraperitoneally), a scavenger of peroxynitrite, significantly attenuated intracranial pressure, cerebrospinal fluid white blood cell count, and blood-brain barrier leakage, as indicated by Evans blue concentration in the cerebrospinal fluid (21.6 ± 9.3 mm Hg, 5776 ± 1790 cells/µL, 9.7 ± 6.4 µg/mL in infected, untreated rats vs. 7.2 ± 1.6 mm Hg, 2004 ± 904 cells/µL, 1.1 ± 1.0 µg/mL infected, uric acid–treated rats, mean ± SD, P < .05). These data suggest that peroxynitrite plays a central role in mediating pathophysiological alterations during bacterial meningitis.

Despite effective antibiotic therapy, pneumococcal meningitis is still a serious disease, with mortality rates exceeding 20% [1]. Among the complications that contribute to the unfavorable clinical outcome during the acute phase of bacterial meningitis are brain edema and increased intracranial pressure (ICP). Experimental studies have identified reactive oxygen species (ROS) [2] and nitric oxide (NO) [3, 4] as key mediators in the pathophysiology of bacterial meningitis. Both contribute to the development of increased ICP, blood-brain barrier disruption, and cerebrospinal fluid (CSF) leukocytosis. Superoxide (O$_2^-$) and NO can react to form peroxynitrite (ONOO$^-$) [5], a strong oxidant that exerts its cytotoxic effects by various mechanisms, including DNA damage, tyrosine nitration, and lipid peroxidation [6, 7]. Peroxynitrite seems to play a role in various disorders of the brain and spinal cord, such as multiple sclerosis [8], cerebral ischemia [9], and familial amyotrophic lateral sclerosis [10], as well as in systemic inflammatory disease, for example, in the heart and the lung of septic patients [11, 12]. We therefore investigated the involvement of peroxynitrite in the pathophysiology of bacterial meningitis.

Peroxynitrite was detected by immunohistochemistry, by use of an anti-3-nitrotyrosine antibody [13] in a well-characterized rat model of bacterial meningitis. The pathophysiological role of this strong oxidant was investigated by treating the animals with uric acid, a natural scavenger of peroxynitrite [14].

Methods

Animal model of pneumococcal meningitis. The experiments were performed by use of a well-characterized rat model of meningitis [15]. Briefly, adult male Wistar rats (300–350 g) were anesthetized with halothane (Hoechst AG, Frankfurt, Germany), and meningitis was induced by transcutananeous intracisternal injection of 150 µL of 10$^7$ colony-forming units/mL of Streptococcus pneumoniae type 3 [3]. Each rat was put into an individual cage and allowed to wake up. Twenty-four hours after infection, the animals were anesthetized (thiopental 100 mg/kg intraperitoneally), tracheotomized, and artificially ventilated with a small animal ventilator (Model Ap-10; Effenberger, Pfaffing, Germany). Body temperature was monitored with a rectal thermometer. A catheter was inserted into the left femoral artery for continuous monitoring of mean arterial blood pressure (MABP) and for blood gas and hematocrit analyses. The left femoral vein was cannulated for Evans blue administration to evaluate blood-brain barrier permeability [16, 17]. A catheter was introduced into the cisterna magna for continuous ICP monitoring and to determine CSF white blood cell (WBC) counts. One hour after intravenous injection of 1 mL of 1% Evans blue, the rats were perfused with 150 mL of ice-cold PBS (pH 7.4), after which the brain was removed and stored at −80°C.

Animals were either uninfected (injected intracisternally with 150 µL PBS, n = 5, controls), infected (n = 5), or infected and injected intraperitoneally with uric acid (300 mg/kg, n = 5) immediately before infection.

Immunohistochemistry for 3-nitrotyrosine. Immunohistochemistry was performed as described by Ischiropoulos et al. [18]. Briefly, 10 µm cryostat-cut sections were incubated with 100% ethanol for 30 min for fixation, and then endogenous peroxidases were quenched by incubating slices with 0.3% methanolic hydrogen per-
Physiological and pathophysiological variables. Induction of meningitis caused a significant ($P < .05$) increase of ICP (21.6 ± 9.3 vs. 1.4 ± 0.9 mm Hg in controls, figure 1), CSF-WBC count (5776 ± 1790 vs. 150 ± 178 cells/µL in controls, figure 2), CSF Evans blue concentration (9.7 ± 6.4 vs. 0.2 ± 0.2 µg/mL in controls, figure 3), body temperature (38.1°C ± 0.4°C vs. 36.9°C ± 0.1°C in controls), and a decrease in MABP (63 ± 24 vs. 118 ± 9 mm Hg in controls). Treatment of rats with uric acid significantly ($P < .05$) reduced ICP (7.2 ± 1.6 vs. 21.6 ± 9.3 mm Hg in infected, untreated rats, figure 1), CSF-WBC count (2004 ± 904 vs. 5776 ± 1790 cells/µL in infected, untreated rats, figure 2), Evans blue extravasation (1.1 ± 1.0 vs. 9.7 ± 6.4 µg/mL in infected, untreated rats, figure 3), and body temperature (37.1°C ± 0.5°C vs. 38.1°C ± 0.4°C in infected, untreated rats). MABP was not significantly influenced by uric acid treatment (72 ± 22 vs. 63 ± 24 mm Hg in infected, untreated rats).

Immunohistochemical studies of brain sections. Brains were sectioned and stained with antinitrotyrosine antibody. Staining was detected in the leptomeninges and the neighboring brain parenchyma of infected, untreated rats. Immunoreactivity was maximal around meningeal blood vessels (figure 4A) and in
close proximity to inflammatory cells in the subarachnoid space (figure 4B). Staining was almost completely reduced by preincubation of the primary antibody with 3-nitrotyrosine (figure 4C), and no nitrotyrosine immunoreactivity was observed when the primary antibody was omitted (figure 4D). Treatment with uric acid substantially reduced nitrotyrosine immunoreactivity (figure 4E) compared with that in infected, untreated animals. Brains from controls did not stain for nitrotyrosine (figure 4F).

Localization of blood-brain barrier disruption. Evans blue extravasation was most pronounced in the leptomeninges and, to a lesser degree, in the brain parenchyma and was colocalized to nitrotyrosine immunoreactivity (figure 5A). Blood-brain barrier breaching was greatly reduced in infected, uric acid–treated rats (figure 5B). No Evans blue fluorescence was detectable in controls (figure 5C).

Discussion

The major findings of this study are (1) the demonstration of peroxynitrite formation within the leptomeninges during experimental bacterial meningitis and (2) the beneficial effects of the peroxynitrite scavenger uric acid on alterations of ICP, CSF-WBC count, and blood-brain barrier disruption in a rat model of pneumococcal meningitis.

Recent experimental studies have clearly shown that ROS and reactive nitrogen intermediates play a crucial role in the pathophysiology of bacterial meningitis [20]. In particular, ROS generation has been detected in brain sections of infant rats with group B streptococcal meningitis by use of the manganese/diaminobenzidine method [21] and in a rat model of pneumococcal meningitis by use of the in vivo lucigenin-enhanced chemiluminescence technique [22]. Furthermore, several antioxidants attenuated meningitis-associated intracranial complications (such as increase of ICP, blood-brain barrier permeability, and brain water content) and tissue injury in experimental bacterial meningitis [3, 23–25]. Like free oxygen radical scavengers, a variety of nonselective NO-synthase inhibitors were shown to alleviate pathophysiological changes such as brain edema formation, blood-brain barrier disruption, and meningeval inflammation in experimental models of bacterial meningitis [3, 4, 26]. In addition, clinical and experimental studies report an increase in NO/nitrite levels in the CSF during bacterial meningitis [3, 4, 27–29].

ROS and NO can react to form the powerful oxidative agent peroxynitrite. Peroxynitrite, at physiological pH, is a short-lived toxin, but the nitrated tyrosine residues can be stable for years [6]. Nitrotyrosine (NT) residues are a widely accepted marker of peroxynitrite [6], and the antibodies to NT-containing proteins that have been raised by Beckman et al. [30] have been used to identify peroxynitrite in various neurological diseases, as well as in systemic inflammatory conditions. For example, NT immunoreactivity was detected in lung tissue from patients with sepsis and pneumonia [12] and in the myocardium of patients with myocarditis and sepsis [11]. NT was detected in the human brain in and around multiple sclerosis plaques [8], and in experimental allergic encephalomyelitis positive NT staining could be localized to microglia/macrophages [31]. NT was also detected in focal ischemic lesions of the brain [32], where it was localized to polymorphonuclear cells [9].

Using immunohistochemistry, we found l-nitrotyrosine positive staining in brain sections of adult rats infected with S. pneumoniae but not in control rats. In our study, nitrotyrosine immunoreactivity was observed in the leptomeninges, especially perivascularly and in close proximity to inflammatory cells in the subarachnoid space, and the neighboring brain parenchyma. The restriction of nitrotyrosine staining to the leptomeninges, perivascularly and around inflammatory cells, indicates that granulocytes, and perhaps cells of the vessel wall, such as smooth muscle cells or endothelial cells, are the most probable source of NO and ROS that react to peroxynitrite.

In recent in vitro studies using a cell culture approach and lucigenin-enhanced chemiluminescence, we have shown that polymorphonuclear leukocytes (PMNL) and cerebromicrovascular endothelial cells produced ROS after stimulation with S. pneumoniae [20]. Likewise, when stimulated with pneumococcal cell-wall components, cerebromicrovascular endothelial cells and rat PMNL were found to release NO/nitrite into the cell-culture supernatants [3, 33]. In an infant rat model of meningitis due to group B streptococci, superoxide production (using the manganese/diaminobenzidine method) was found to be colo-
Figure 4. Ten micrometer cryostat brain sections of rats 24 h after intracisternal injection of either live pneumococci (A–E) or PBS (controls, F) were stained with an antinitrotyrosine antibody, by use of peroxidase and 3-amino-9-ethylcarbazole chromogen as detection system. Specific staining yields a red reaction product. Counterstaining was performed with hematoxylin. (A) Perivascular nitrotyrosine immunoreactivity in meningeal blood vessel in an infected, untreated animal. (B) Nitrotyrosine staining in close proximity to inflammatory cells of subarachnoid space and in neighboring brain tissue. (C) Staining is almost completely abolished by preincubation of nitrotyrosine antibody with 10 mM 3-nitrotyrosine. (D) No specific staining can be detected when primary antibody is omitted. (E) Substantial reduction of nitrotyrosine immunoreactivity in an animal treated with uric acid, a natural scavenger of peroxynitrite, before induction of meningitis. (F) No staining is observed in uninfected animals. Magnification bar in A equals 50 μm for A–F.
Figure 5. Fluorescence microscopy of 10 μm cryostat brain sections. Animals were injected intravenously with Evans blue before being killed. Extravasation of dye, reflecting sites of blood-brain barrier disruption, was observed under green fluorescence microscopy (excitation filter 545 nm, barrier filter 590 nm), appearing red, and was photographed with a black and white video camera system. (A) Blood-brain barrier breaching is localized predominantly in the subarachnoid space in an infected, untreated rat. (B) Blood-brain barrier disruption is greatly reduced in infected rats that have been pretreated with uric acid, a natural scavenger of peroxynitrite. (C) In uninfected animals no extravasation of Evans blue occurs.

calized with PMNL in the subarachnoid and ventricular space and also along penetrating cortical vessels [21], suggesting that cells other than PMNL, such as endothelial cells, contribute to ROS production in bacterial meningitis. Other potential sites of NO and ROS generation during bacterial meningitis may include microglia, neurons, and astrocytes. In vitro, when stimulated with pneumococcal cell-wall components, microglial cells, astrocytes, and cerebellar neurons were shown to release NO/nitrite in the cell-culture supernatants [3, 34, 35]. In contrast, murine neuron-like cells and primary rat astrocytes did not generate ROS when stimulated with pneumococci [36]. Furthermore, no superoxide production was detected in neurons and astrocytes when brain sections from infant rats with meningitis due to group B streptococci were stained with manganese/diaminobenzidine [21]. The definite cellular source of peroxynitrite during meningitis still needs to be determined.

Uric acid is a widely accepted scavenger of peroxynitrite [37]. In vitro, uric acid dose-dependently blocks peroxynitrite-mediated oxidation of dihydrorhodamine without affecting NO production from macrophages stimulated with lipopolysaccharide [38]. In vivo, uric acid has been shown to be protective in experimental allergic encephalomyelitis by targeting peroxynitrite [14]. Our study has shown that treatment of pneumococci-infected rats with uric acid significantly reduced ICP, CSF-WBC count, and blood-brain barrier breaching compared with infected, untreated animals. This suggests that peroxynitrite contributes to the development of intracranial complications in bacterial meningitis, such as brain edema and meningeal inflammation. Peroxynitrite can exhibit various toxic effects, including tyrosine nitration, interference with key enzymes of the tricarboxylic acid cycle, the mitochondrial respiratory chain, mitochondrial calcium metabolism, lipid peroxidation, induction of DNA single-strand breakage, activation of matrix metalloproteinases (MMPs) from their inactive pro-enzyme forms, and inactivation of their natural opponents, the tissue inhibitors of MMPs [6, 7, 39-42]. These mechanisms of action of peroxynitrite may also apply to bacterial meningitis and contribute to oxidative brain damage, blood-brain barrier disruption, and meningeal inflammation. Recent studies provided evidence for the involvement of MMPs in the pathophysiology of bacterial meningitis. Thus, (1) MMP-9 activity was found in the CSF of patients with bacterial meningitis (but not in controls), (2) MMP-9 activity was also found in the CSF of rats with experimental meningococcal meningitis, and (3) treatment with the MMP inhibitor batimastat reduced meningitis-associated blood-brain barrier disruption and CSF pleocytosis in experimental meningococcal meningitis [16]. Lipid peroxidation by peroxynitrite causes loss of membrane function and integrity [43]. Lipid peroxidation products have been detected in the rat brain in experimental bacterial meningitis [21] and in the CSF of patients with bacterial meningitis [20]. Moreover, treatment with the lipid peroxidation blocker U74389F was beneficial in experimental meningitis [44]. Lipid peroxidation is therefore another potential clue to the protective effect of the peroxynitrite scavenger uric acid. Peroxynitrite-induced DNA single-strand breaks can lead to the activation of poly(ADP-ribose) polymerase (PARP) [7, 42]. Ultimately, PARP activation depletes cells of energy, causing necrotic cell death. In experimental bacterial meningitis, we (U. Koedel, unpublished observations) and others [21] have observed necrotic cortical injury. The PARP inhibitor 3-aminobenzamide has been shown to reduce meningitis-associated cerebrovascular alterations, blood-brain barrier breaching, and meningeal inflammation in an adult model of pneumococcal meningitis [45]. Thus, PARP offers another link between peroxynitrite and the pathophysiological alterations and meningitis-associated brain damage during the course of bacterial meningitis.

In conclusion, we have shown peroxynitrite formation im-
munohistochemically in the brain of rats with experimental pneumococcal meningitis. Scavenging peroxynitrite by uric acid reduced CSF leukocytosis, ICP, and blood-brain barrier disruption, suggesting that this strong oxidant is a central mediator in the pathophysiology of bacterial meningitis.

References


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