VIROLOGY

Inducible nitric oxide synthase inhibition delays death of rabies virus-infected mice

SUKATHIDA UBOL, CHAREEPORN SUKWATTANAPAN and YAOWAPA MANEERAT*

Department of Microbiology, Faculty of Science and *Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

A pathophysiological mechanism of cerebral damage and impairment of neuronal function during rabies virus infection was examined. Synthesis of nitric oxide (NO) and expression of the inducible nitric oxide synthase (iNOS) gene are strongly upregulated during rabies virus infection. Treatment of rabies virus-infected mice with a selective inhibitor of iNOS, aminoguanidine (AG), significantly delayed their death. Prolonged survival was not due to suppression of an inflammatory response in the central nervous system. One effect of iNOS inhibition was at the level of viral replication. Treatment with AG delayed rabies virus replication by 2 days. Moreover, iNOS inhibition also suppressed an early phase of expression of an apoptotic gene, Caspase-1, which resulted in slow progression of infected cells into apoptotic death. iNOS inhibition had no effect on expression of the anti-apoptotic gene, *bcl-2*. In conclusion, iNOS inhibition delayed the death of rabies virus-infected mice by affecting viral replication and apoptotic death of infected cells.

Introduction

Nitric oxide (NO) is a short-lived diffusible molecule, the production of which is catalysed by nitric oxide synthase (NOS) [1]. Two major isoforms of NOS, inducible and constitutive, have been identified. Constitutive NOS, cNOS, is present in endothelial cells and neurons and catalyses low-level NO production, which plays a role in neurotransmission and vasodilatation [2, 3]. Inducible NOS, iNOS, is induced as a response to various stimuli such as endotoxins and inflammatory cytokines. [4]. NO synthesis due to iNOS has been reported to be involved in the pathogenesis of neurodegenerative diseases [5–7]. For example, experimental auto-immune encephalitis in mice is a demyelinating disease associated with an excessive production of NO [8].

Rabies is a disease of the central nervous system which involves significant upregulation of iNOS gene expression and downregulation of neuronal cNOS (nNOS) gene expression [9]. The role of NO in the pathogenesis of rabies is as yet unclear, but is likely to be multifaceted. NO may play a role in blood–brain barrier leakage and may contribute to tissue damage

due to the generation of peroxynitrite [9, 10]. NO by itself has been shown to induce neural cell death *via* apoptosis and replication of rabies virus in neural cells induces apoptotic cell death *in vivo* and *in vitro* [7, 11, 12]. Moreover, NO also influences the replication kinetics of many viruses [13, 14]. To obtain a better understanding of the potential role of NO in the pathogenesis of rabies, the present study examined whether NO production influences the apoptotic death of neurons, the expression of apoptotic genes and the kinetics of rabies virus replication. The study also investigated whether, if NO exerts such effects, inhibition of NO production could alter the survival time of rabies virus-infected mice. The aim of the study was to increase understanding of the pathogenesis of rabies encephalitis.

Materials and methods

Viruses

A fixed strain of rabies virus (CVS-11) was obtained from the Queen Soavabha Memorial Institute, Bangkok, Thailand. The stock virus was prepared as a 10% mouse brain suspension as follows. CVS-11-infected brains were homogenised, prepared as 10% brain suspension in D-MEM and clarified by centrifugation at 10 000 rpm (IEC-B-22M, International Equipment Company, USA) at 4°C for 20 min. The clarified brain suspension was divided into small volumes and stored

at -70°C until used. Stock virus suspension was assayed by fluorescent foci staining on N18 cells and had titres of $c. 6 \times 10^6$ FFU/ml.

Determination of the effect of iNOS inhibition on survival time of rabies virus-infected mice

Adult Swiss albino mice were inoculated with 50 μl of CVS-11 at a concentration of 6×10^5 ffu/ml and treated with one of two specific NOS inhibitors: 7-nitroindazole (7-NI; an nNOS inhibitor; Sigma) or aminoguanidine (AG; an iNOS inhibitor; Sigma). nNOS was blocked by intraperitoneal injection of 7-NI in alcohol 10% at concentrations of 15 mg/kg ($n = 12$) or 30 mg/kg ($n = 18$) twice a day; the control mice were given alcohol 10% in distilled water ($n = 18$) as described by Mackenzie *et al.* [15]. For iNOS inhibition, mice were inoculated twice a day with AG at concentrations of 400 mg/kg ($n = 10$) or 800 mg/kg ($n = 10$); the control group was treated with PBS ($n = 10$) as reported by Cross *et al.* [8]. Each group of mice was maintained with free access to food and survival time was observed and expressed as mean (SD) hours. Differences in survival time between specific isoform inhibitor treatment groups and control groups were analysed statistically by an unpaired *t*-test at confidence intervals of 95% ($p < 0.05$) and 99% ($p < 0.01$).

Determination of the effect of iNOS inhibition on rabies virus-induced apoptotic death

Adult mice were inoculated with 50 μl of CVS-11 at a concentration of 6×10^5 FFU/ml *via* the footpad and then treated with AG 800 mg/kg twice a day for 7 days; control mice were treated with PBS. Mice were killed and brains were collected at various times. These samples were used to study the kinetics of rabies virus replication by LD50 assay, to investigate apoptosis by the in-situ TUNEL technique and to determine apoptotic gene expression by reverse transcription (RT)-PCR.

To study the kinetics of rabies virus replication, the titres of virus in brains from AG-treated and control mice at 0, 1, 2, 4, 5, 6 and 7 days after infection were determined by mouse inoculation. The LD50 titre was calculated by the method of Reed and Muench [16].

Apoptosis detection by in-situ TUNEL assay

Brains from AG-treated and control mice were fixed in formalin and embedded in paraffin. The paraffin sections were deparaffinised and rehydrated in an alcohol series. Nuclear DNA fragmentation of cells in these brain sections was detected by the TUNEL technique (Boehringer Mannheim, Mannheim, Germany). The TUNEL reaction mixture consisted of terminal deoxynucleotidyl transferase (TdT) and fluorescein-labelled dUTP. TdT was used for the incorpora-

tion of fluorescein-dUTP to DNA strand breaks *in situ*. The fluorescein signal was amplified by staining with anti-fluorescein-conjugated alkaline phosphatase. Positive signals were developed by adding a substrate containing 4-nitroblue tetrazolium chloride and 5-bromo-3-indolyl-phosphate (Boehringer Mannheim). Apoptotic nuclei were identified by light microscopy.

Determination of Caspase 1 (ICE) and bcl-2 mRNA expression in mouse brains by RT-PCR

Infected mouse brains from both the AG-treated (800 mg/kg) and PBS-treated groups were used to study the expression of the genes involved in neuronal cell death, ICE and *bcl-2*, by RT-PCR. Mouse brains were homogenised in RNAase-free PBS. Total RNA was isolated from homogenised brain with TRIZOL (Life Technologies, Grand Island, NY, USA) as recommended by the manufacturer. A portion of the extracted RNA was subjected to the RT-PCR procedure as described previously [17].

Determination of the effect of iNOS inhibition on intracerebral inflammation

Brains were harvested from rabies virus-infected mice treated and untreated with AG. These brains were fixed with formalin, embedded, sectioned and stained with haematoxylin and eosin. The degrees of lymphocytic infiltration were compared.

Results

Effect of iNOS inhibition on survival of rabies virus-infected mice

To determine the effect of NO synthesis due to iNOS on the pathogenesis of rabies, infected mice were treated with an iNOS inhibitor (AG). The survival time of AG-treated mice was compared with that of mice treated with an nNOS inhibitor (7-NI) or diluent. Treatment with AG 400 mg/kg dramatically suppressed iNOS activity, detected by quantifying the amount of [^3H] L-citrulline produced from [^3H]L-arginine by NOS in mouse brain homogenates (data not shown). For rabies virus-infected mice, there was no difference in mean survival time between the 7-NI-treated and control groups ($p > 0.05$) (Table 1). In contrast, the survival time of the AG-treated group increased and

Table 1. The effect of NOS inhibition on the survival time of rabies virus-infected mice

Treatment group	Number of mice	Mean (SD) survival time (h)	p value
alcohol 10%	18	184.80 (14.70)	—
7-NI 15 mg/kg	12	183.40 (8.90)	$>0.05^*$
7-NI 30 mg/kg	18	181.83 (16.70)	>0.05
PBS	10	168.26 (21.17)	—
AG 400 mg/kg	10	193.10 (21.53)	$<0.05^*$
AG 800 mg/kg	10	205.53 (34.50)	$<0.01^\dagger$

Unpaired *t* test at confidence levels of $^*95\%$ and $^\dagger99\%$.

was dose-dependent. Treatment with AG 400 mg/kg significantly delayed the time to death (193.10, SD 21.53, h; $p < 0.05$) and this effect was more evident with 800 mg/kg (205.53, SD 34.50, h; $p < 0.01$).

Effect of iNOS inhibition on the kinetics of rabies virus replication in mouse brain

To determine whether the slowing of disease progression in AG-treated mice was due to the effect of NO on rabies virus replication, the kinetics of rabies virus replication in AG-treated mice and control mice were compared. Replication of rabies virus in the control group increased rapidly and peaked on day 5, whereas replication in the AG-treated group peaked on day 6. The onset of viral replication in the AG-treated group was delayed by 24–48 h compared with the control group (Fig. 1). This time lag corresponded to the extension of survival time.

Correlation between inhibition of iNOS activity and progression of apoptotic death in rabies virus-infected mouse brain

To determine whether NO resulting from iNOS activity played a role in apoptotic cell death during rabies virus infection *in vivo*, the morphological changes of apoptotic nuclei in rabies virus-infected mouse brain cells were studied. By TUNEL staining, apoptotic nuclei could be detected as early as day 4 after infection, but the signal and number of apoptotic cells were too low to differentiate between treated and untreated groups (data not shown). By day 5, apoptosis was higher in control mice than in AG-treated mice (Fig. 2). The TUNEL-positive nuclei were found in both the cerebellum and the brainstem of the infected brains.

Fig. 1. Effect of inhibition of NO production by AG treatment (800 mg/kg) on rabies virus replication. LD50 titres of infected brains of AG-treated and PBS-treated mice are shown as means (SD) at 0, 1, 2, 4, 5, 6 and 7 days after infection. ●, control group; ■, AG-treated group.

Fig. 2. TUNEL assay. In-situ detection of apoptotic cells in rabies virus-infected brains of AG (800 mg/kg)-treated mice (b and d), PBS-treated mice (a and c) and uninfected control mice (e and f) at 5 days after infection. Magnification: $\times 100$ for a, b, and e, $\times 200$ for c, d and f.

The effect of iNOS inhibitor on ICE and bcl-2 gene expression in rabies virus-induced apoptosis

To study the mechanism of delayed apoptotic death following iNOS inhibition, expression of the apoptotic gene, ICE and of the anti-apoptotic gene, bcl-2, were monitored by semi-quantitative RT-PCR of the brains of infected mice treated with AG 800 mg/kg or PBS. With the β -actin gene as an internal control, the expression of bcl-2 was no different in the two groups. Furthermore, the level of bcl-2 gene expression did not change during the course of infection (Fig. 3). Rapid activation of ICE was detected in both groups of mice.

Fig. 3. Expression of the bcl-2 gene in rabies virus-infected brains of PBS-treated (A) and AG- (800 mg/kg) treated mice (B) at 3, 5 and 7 days after infection as measured by semi-quantitative RT-PCR. The amount of β -actin mRNA served as an internal control.

By days 4 and 5 after infection, expression of ICE was significantly lower in AG-treated mice than in the PBS-treated group. However, by day 7 the level of ICE expression in both groups was the same (Fig. 4). The data demonstrate that inhibition of NO production suppresses ICE gene expression in rabies virus-infected mouse brain, but has no effect on *bcl-2* gene regulation.

Effect of AG on intracerebral inflammatory response

The prolonged survival time of AG-treated, rabies virus-infected mice could result from an inhibitory effect of AG on the inflammatory response leading to a reduction of neuropathological lesions. Therefore, the degree of CNS inflammation was compared in AG-treated and untreated groups. Inflammation in rabies virus-infected brain was not suppressed significantly by AG treatment (Table 2).

Discussion

This study demonstrated that inhibition of NO production by an iNOS inhibitor significantly increased the survival time of rabies virus-infected mice. Extension of the survival time was accompanied by delayed viral replication, slow progression to apoptotic death and delayed expression of the apoptotic gene, ICE, but

Table 2. Comparison of inflammatory response between rabies virus-infected mice treated with AG and untreated

Group	Days after infection				
	1	2	3	4	5
Infected mice	+	+/++	++/+++	++	ND
Infected/treated mice	+	++	++	++	+/++

+, few inflammatory cells in congested blood vessels and infiltrates in brain tissues; ++, moderate numbers of inflammatory cells in blood vessels and tissues; +++, large numbers of inflammatory cells (particularly lymphocytes) forming focal inflammation in brain tissue.

there was no effect on the intracerebral inflammatory response. The significance of iNOS inhibition, but not nNOS inhibition, on the severity of rabies virus infection is supported by the evidence that only iNOS mRNA is dramatically upregulated during rabies virus infection [9]. The role of NO in the pathogenesis of CNS disease is apparently paradoxical. This gaseous molecule can be neuroprotective as well as neurodestructive [18]. A pathogenic role of NO has been demonstrated in viral infections of the CNS. For example, the degree of neurocognitive defect (dementia) during HIV infection correlates with iNOS expression [19]. Furthermore, NO has been shown to play a role in breakdown of the blood–brain barrier in central nervous system complications of HIV infections [20]. The data from the present study on increased survival time of rabies virus-infected mice after iNOS inhibition support the proposition of a pathogenic role for NO during rabies virus infection.

It is established that NO is involved in the apoptotic cell death of neurons [7]. Whether apoptotic death of infected neurons acts as a defence mechanism is unclear. It is possible that an appropriate level of cell death may function to reduce the number of virus-infected cells. However, excessive or massive death of such a non-regenerating population may contribute to disease progression. Hooper *et al.* have reported that during rabies virus infection the production of NO increases *c.* 30-fold [10]. NO is a diffusible molecule and such a high level of NO may contribute to the death of neurons. The argument for NO as a neurotoxin or neuroprotectant is based on its oxidative/reductive status. The oxidised state, NO^+ , is proposed to mediate neuroprotective effects through S-nitrosylation of thiol groups. The reduced state, $\text{NO}^\bullet$, reacts with superoxide, O_2^- , to form peroxynitrite (ONOO^-), a highly reactive molecule that can initiate non-specific protein and lipid peroxidation. It is possible that the reduced state of NO is dominant in rabies virus-infected brains.

AG treatment delayed the onset of rabies virus growth *in vivo*. This suggests that NO accelerates rabies virus replication. Most publications report an inhibitory effect of NO on replication of both DNA and RNA viruses, e.g., NO inhibits vaccinia virus replication by blocking viral DNA and late protein synthesis [21]. In

Fig. 4. The level of ICE mRNA expression in rabies virus-infected brains of AG (800 mg/kg) -treated and PBS-treated mice as determined by semi-quantitative RT-PCR. Mean levels of ICE mRNA relative to levels of β -actin mRNA from PBS-treated and AG-treated mouse brains at 0, 4, 5 and 7 days after infection are presented. ●, control group, ■, AG-treated group.

Epstein-Barr virus (EBV) infection, NO inhibits DNA synthesis and blocks activation of the latent EBV genome [14]. For Sindbis virus infection, it was hypothesised that NO may inhibit virus maturation by inhibition of structural protein palmitoylation [13]. The mechanism by which NO could enhance viral replication has not been elucidated. However, immediate early response genes such as *c-fos* and *junB* are strongly upregulated in rabies virus-infected brain tissue [22]. The function of Fos and Jun are redox-regulated [23]. NO, or reaction of NO with other reactive oxygen intermediates, has been shown to regulate redox(-sensitive) signalling pathways [24]. Delayed replication of rabies virus in the presence of an iNOS inhibitor may result from the effect of NO on the function of *c-fos*. Another possible explanation for delayed onset of rabies virus replication during AG treatment is that treatment resulted in an as yet unidentified impairment of host cell activity.

In summary, the present study indirectly demonstrated possible effects of NO on the pathogenesis of rabies encephalitis. This diffusible molecule may directly induce the apoptotic death of neurons. The other possibility was that NO directly accelerated rabies virus replication which then subsequently caused death of infected neurons.

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References

- Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; **43**: 109–142.
- Garthwaite J, Charles SL, Chess-Williams R. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* 1988; **336**: 385–388.
- Dawson VL, Dawson TM. Physiological and toxicological actions of nitric oxide in the central nervous system. *Adv Pharmacol* 1995; **34**: 323–342.
- Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992; **6**: 3051–3064.
- Schulz JB, Matthews RT, Beal MF. Role of nitric oxide in neurodegenerative diseases. *Curr Opin Neurol* 1995; **8**: 480–486.
- Ikonomidou G, Turski L. Exitotoxicity and neurodegenerative diseases. *Curr Opin Neurol* 1995; **8**: 487–497.
- Kroncke K-D, Fehsel K, Klotzbach V. Nitric oxide: cytotoxicity versus cytoprotection – how, why, when and where? *Nitric Oxide* 1997; **1**: 107–120.
- Cross AH, Misko TP, Lin RF, Hickey WF, Trotter JL, Tilton RG. Aminoguanidine, an inhibitor of inducible nitric oxide synthase, ameliorates experimental autoimmune encephalomyelitis in SJL mice. *J Clin Invest* 1994; **93**: 2684–2690.
- Akaiki T, Weihe E, Schaefer M *et al*. Effect of neurotropic virus infection on neuronal and inducible nitric oxide synthase activity in rat brain. *J Neurovirol* 1995; **1**: 118–125.
- Hooper DC, Ohnisi ST, Kean R, Numagami Y, Dietzchold B, Koprowski H. Local nitric oxide production in viral and autoimmune disease of the central nervous system. *Proc Natl Acad Sci USA* 1995; **92**: 5312–5316.
- Jackson AC, Rossiter JP. Apoptosis plays an important role in experimental rabies virus infection. *J Virol* 1997; **71**: 5603–5607.
- Theerasurakarn S, Ubol S. Apoptosis induction in brain during the fixed strain of rabies virus infection correlates with onset and severity of illness. *J Neurovirol* 1998; **4**: 407–414.
- Tucker PC, Griffin DE, Choi S, Bui N, Wesseling S. Inhibition of nitric oxide synthesis increases mortality in Sindbis virus encephalitis. *J Virol* 1996; **70**: 3972–3977.
- Kawanishi M. Nitric oxide inhibits Epstein-Barr virus DNA replication and activation of latent EBV. *Intervirology* 1995; **38**: 206–213.
- MacKenzie GM, Rose S, Bland-Ward PA, Moore PK, Jenner P, Marsden CD. Time course of inhibition of brain nitric oxide synthase by 7-nitro indazole. *Neuroreport* 1994; **5**: 1993–1996.
- Blake K, O'Connell S. Virus culture. In: Harper DR (ed) *Virology*. LabFax. Oxford, Blackwell Scientific Publications. 1993; 81–122.
- Ubol S, Sukwattanapan C, Utasinchaoen P. Rabies virus replication induces Bax-related, caspase dependent apoptosis in mouse neuroblastoma cells. *Virus Res* 1998; **56**: 207–215.
- Lipton SA, Choi Y-B, Pan Z-H *et al*. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 1993; **364**: 626–632.
- Adamson DC, Wildemann B, Sasaki M *et al*. Immunologic NO synthase: elevation in severe AIDS dementia and induction by HIV-1 gp41. *Science* 1996; **274**: 1917–1921.
- Giovannoni G, Miller RF, Heales SJ, Land JM, Harrison MJ, Thompson EJ. Elevated cerebrospinal fluid and serum nitrate and nitrite levels in patients with central nervous system complications of HIV-1 infection; a correlation with blood-brain-barrier dysfunction. *J Neurol Sci* 1998; **156**: 53–58.
- Mělková Z, Esteban M. Inhibition of Vaccinia virus DNA replication by inducible expression of nitric oxide synthase. *J Immunol* 1995; **155**: 5711–5718.
- Fu ZF, Weihe E, Zheng YM *et al*. Differential effects of rabies and Borna disease viruses on immediate-early- and late-response gene expression in brain tissues. *J Virol* 1993; **67**: 6674–6681.
- Abate C, Patel L, Rauschere FJ, Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. *Science* 1990; **249**: 1157–1161.
- Stamler JS. Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* 1994; **78**: 931–936.