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Short Communication

Lack of nitric oxide synthase type 2 (NOS2) results in reduced neuronal apoptosis and mortality following mouse hepatitis virus infection of the central nervous system

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The role of nitric oxide synthase type-2 (NOS2)-derived nitric oxide (NO) in the pathogenesis of mouse hepatitis virus (MHV)-induced central nervous system disease was examined. Infection of NOS2 knockout ($^{-/-}$) and NOS2$^{+/+}$ mice with MHV resulted in similar kinetics of viral clearance from the brain and comparable levels of demyelination. MHV-infected NOS2$^{-/-}$ mice displayed a marked decrease in mortality as compared to infected NOS2$^{+/+}$ mice that correlated with a significant decrease ($P \leq 0.001$) in the number of apoptotic cells (determined by TUNEL staining) present in the brain. Confocal microscopy revealed that the majority of cells (>70%) undergoing apoptosis were neurons. These studies indicate that NOS2-generated NO contributes to apoptosis of neurons but not demyelination following MHV infection. *Journal of NeuroVirology* (2002) **8,** 58–63.

Keywords: nitric oxide; virus; neuroimmunology; demyelination; apoptosis

Introduction

Nitric oxide synthase-2 (NOS2) is the Ca^{2+}-independent, inducible isoform of the NOS family with activity that results in high level expression of the free radical nitric oxide (NO) over an extended period of time (reviewed in MacMicking *et al*, 1997b). NOS2 is expressed by a variety of cell types including macrophage/microglia and astrocytes following exposure to various signals, including inflammatory cytokines such as IFN-γ and TNF-α. As such, numerous studies have determined that NOS2-generated NO is an important component in host defense against microbial infection (Adams *et al*, 1990; Karupiah *et al*, 1993; Lane *et al*, 1994; MacMicking *et al*, 1997a; Zaragoza *et al*, 1999). In addition, NOS2-generated NO may contribute to various inflammatory pathologies by exerting a cytostatic and/or cytotoxic effect (Stuehr and Nathan, 1989; Campbell, 1996; Adler *et al*, 1997).

Infection of susceptible mice with MHV results in an acute encephalomyelitis followed by a chronic demyelinating disease with many clinical and histologic similarities to the human demyelinating disease multiple sclerosis (MS) (Houtman and Fleming, 1996; Lane and Buchmeier, 1997a). NOS2 is expressed during both the acute and chronic disease stages of MHV infection suggesting a role for NOS2-derived NO in the pathogenesis of disease (Sun *et al*, 1995; Grzybicki *et al*, 1997; Lane *et al*, 1997b, 1999). A recent study demonstrated that administration of aminoguanidine (AG), a relatively selective inhibitor of NOS2 activity, to MHV-infected mice resulted in reduced demyelination early following infection. However, demyelination was not affected

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at later time postinfection in AG-treated mice, suggesting a transient role for NO in myelin destruction (Lane *et al*, 1999). The current study utilizes mice with a homozygous deletion in the *nos2* gene ($NOS2^{-/-}$) to further characterize the contributions of NOS2-generated NO in MHV-induced CNS disease. The studies presented demonstrate no significant differences in the severity of demyelination between MHV-infected $NOS2^{-/-}$ mice and $NOS2^{+/+}$ mice. However, MHV-infected $NOS2^{-/-}$ mice displayed a marked decrease in mortality that correlated with a significant reduction ($P \leq 0.001$) in numbers of apoptotic neurons within the CNS as compared to infected $NOS2^{+/+}$ mice. Collectively, these data suggest that NOS2-generated NO contributes to acute neurologic disease by inducing apoptosis of neurons but does not have a role in demyelination.

Results and discussion

$NOS2^{+/+}$ (5–6 weeks, C57BL/6, H-2^b background, Jackson Laboratories, Bar Harbor, Maine) and $NOS2^{-/-}$ mice (5–6 weeks, C57BL/6-*Nos2*^{tm1Lau}, Jackson Laboratories) were infected intracranially (i.c.) with 10 plaque-forming units (PFU) of MHV strain V5A13.1 (Dalziel *et al*, 1986). MHV infection of $NOS2^{+/+}$ mice resulted in earlier onset and more severe clinical disease that correlated with a marked increase in mortality as compared to MHV-infected $NOS2^{-/-}$ mice (Figure 1). Differences in mortality

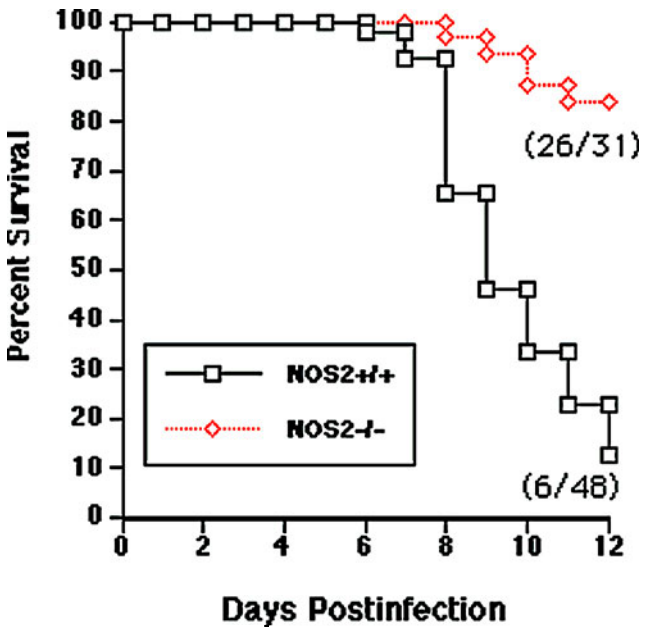

Figure 1 Mortality in MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice. Five- to six-week-old $NOS2^{+/+}$ and $NOS2^{-/-}$ mice were infected intracranially with 10 PFU of MHV-V5A13.1. By day 12 p.i., greater than 85% of MHV-infected $NOS2^{-/-}$ mice had died, whereas only 13% of the $NOS2^{-/-}$ mice died. $NOS2^{+/+}$ mice, $n=55$; $NOS2^{-/-}$ mice, $n=48$.

Table 1 Viral clearance and demyelination in MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice

Mouse	*Days p.i.*	*Titer Log_{10} (PFU/g)*	*n*	*Demyelination*[c]	*n*
$NOS2^{+/+}$	7	6.1 ± 0.5[a]	4	ND[d]	
	12	<2.0[b]	3	2.6 ± 0.3	5
	35	<2.0	2	3.5 ± 0.5	6
$NOS2^{-/-}$	7	5.3 ± 0.5	7	ND	
	12	2.5 ± 0.6	6	2.3 ± 0.4	6
	35	<2.0	2	2.8 ± 0.3	5

[a]All data are shown as mean ± SEM.
[b]Below level of detection of plaque assay (≤100 PFU/g tissue).
[c]Scoring was as follows: 0, no demyelination; 1, mild inflammation accompanied by loss of myelin integrity; 2, moderate inflammation with increasing myelin damage; 3, numerous inflammatory lesions accompanied by significant increase in myelin stripping; and 4, intense areas of inflammation accompanied by numerous phagocytic cells engulfing myelin debris (Lane *et al*, 2000).
[d]Not done.

between the two populations of mice were notable beginning at day 7 p.i. and increased until day 12 p.i., at which point approximately 85% of MHV-infected $NOS2^{-/-}$ mice survived infection as compared to less than 15% survival in the MHV-infected $NOS2^{+/+}$ mice.

The increase in disease severity in $NOS2^{+/+}$ mice was not the result of higher viral titers or delayed clearance of virus from the CNS as infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice displayed comparable viral titers within the brains at all time points examined as assessed by plaque assay (Hirano *et al*, 1978) (Table 1). These data are consistent with previous studies that indicated that NOS2-generated NO does not contribute to clearance of MHV from the CNS (Lane *et al*, 1997b, 1999; Wu *et al*, 2000). To evaluate the severity of demyelination, spinal cords from MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice were removed at days 12 and 35 p.i. Examination of luxol fast blue-stained spinal cords indicated no significant differences in the severity of demyelination between $NOS2^{-/-}$ and $NOS2^{+/+}$ mice at either 12 or 35 days p.i., suggesting that NO does not play an important role contributing to MHV-induced demyelination (Table 1).

Previous studies have shown that NO is capable of triggering events leading to apoptosis (Mebmer *et al*, 1996; Melkova *et al*, 1997; Allione *et al*, 1999). To determine if the diminished disease severity observed in MHV-infected $NOS2^{-/-}$ mice was associated with a reduction in the number of cells undergoing apoptosis, TUNEL staining was performed by FACS analysis (FACStar, Beckton Dickinson, Mountain View, California) on cells obtained from the brains of MHV-infected mice by centrifugation through a discontinuous Percoll gradient (Liu *et al*, 2000). TUNEL analysis was performed using a fluorescein death detection kit (Boehringer Mannheim, Indianapolis, Indiana). FACS analysis of TUNEL-positive cells present within the brains of infected mice revealed that

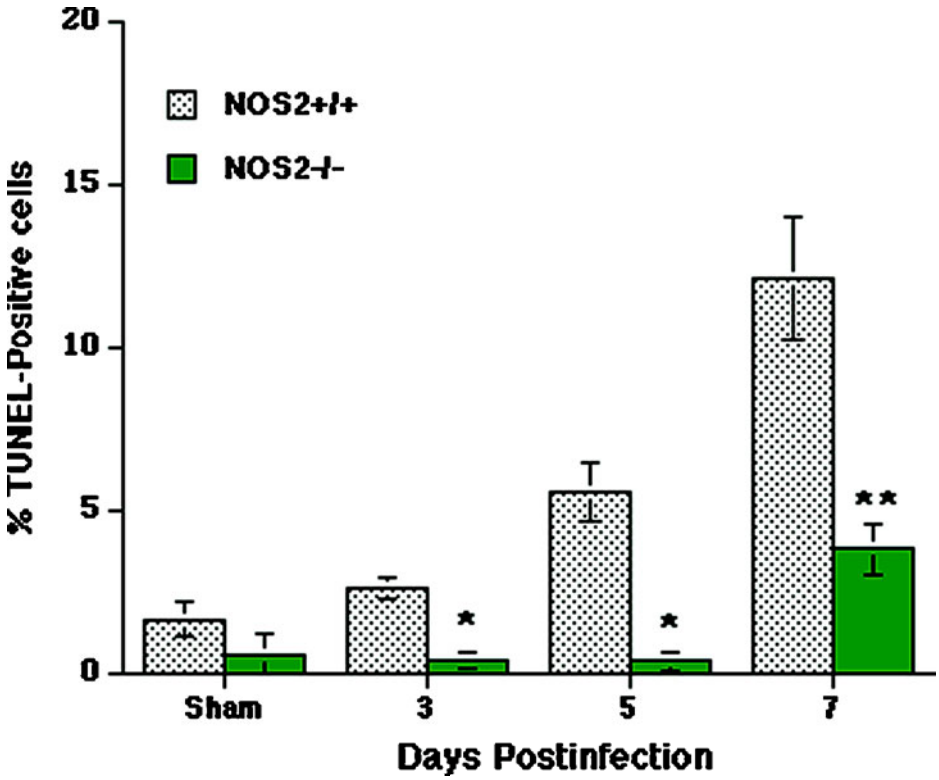

Figure 2 Decreased levels of TUNEL-positive cells within the brains of MHV-infected $NOS2^{-/-}$ mice. MHV-infected $NOS2^{-/-}$ mice exhibited a significant reduction in TUNEL-positive cells present within the CNS when compared to infected $NOS2^{+/+}$ mice at days 3, 5, and 7 p.i. Data reported as mean ± SEM and the results presented represent two separate experiments. For sham mice, $n=4$; day 3 and day 5 p.i., $n=3$; day 7 p.i., $n=8$; * $P \leq 0.04$, ** $P \leq 0.001$.

$NOS2^{-/-}$ mice displayed reduced levels of positive cells at all time points examined (days 3, 5, and 7 p.i.) as compared to infected $NOS2^{+/+}$ mice (Figure 2). Examination of brains of MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice suggested that neurons were damaged and/or destroyed following MHV infection of the CNS. Immunohistochemical staining was performed on 8-μM frozen sections using a mouse monoclonal antibody specific for the neuron-specific nuclear protein NeuN (1:500 dilution, Chemicon, Inc., Temecula, California). Diaminobenzidine was used for a chromagen and neuronal nuclei are stained brown. Such analysis revealed an overall reduction in the thickness and disrupted pattern within the dentate gyrus (DG) combined with the appearance of fragmented neuronal nuclei within the DG and hippocampus of infected $NOS2^{+/+}$ mice (Figure 3). In marked contrast, neurons present within the hippocampus and DG of MHV-infected $NOS2^{-/-}$ mice appeared normal with no apparent damage (Figure 3).

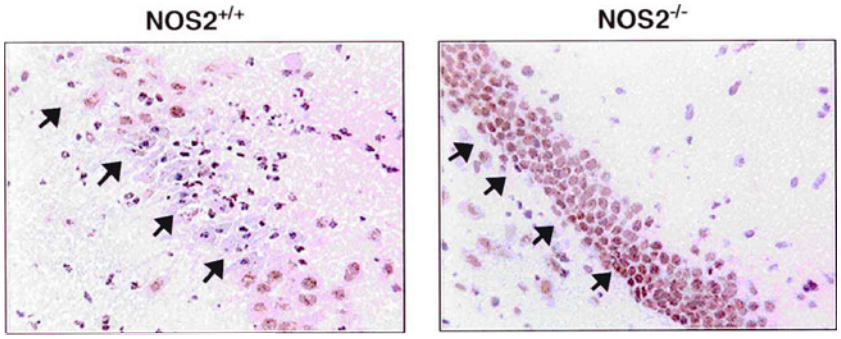

Figure 3 Evidence of neuronal damage in MHV-infected $NOS2^{+/+}$ mice. Shown are representative sections from MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice. Arrowheads indicate neurons (NeuN-positive cells) within the DG. Note the overall reduced thickness and disrupted pattern of the DG from infected $NOS2^{+/+}$ mice as compared to the DG obtained from infected $NOS2^{-/-}$ mice. Original magnification, x200. Control sections with omission of primary antibody were negative. Sections from sham-infected mice treated with primary and secondary antibodies showed healthy neurons (not shown).

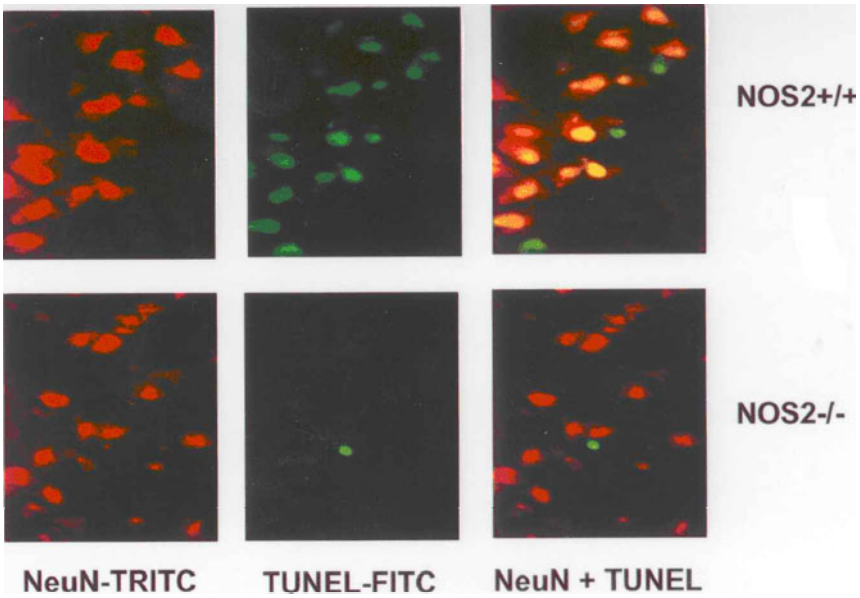

Figure 4 Neurons are TUNEL-positive. Shown are representative sections from infected mice at day 7 p.i., showing neurons (red), TUNEL-positive cells (green), and dual-positive cells (yellow) present within the DG of infected mice. Note the increased number of dual-positive cells in $NOS2^{+/+}$ mice as compared to $NOS2^{-/-}$ mice. Original magnification, x400. Control sections lacking the TdT enzyme and/or NeuN primary antibody were negative in both cases (not shown).

In situ TUNEL analysis was combined with immunofluorescent staining to determine if neurons were undergoing apoptosis within the brains of MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice. Confocal microscopy (Bio-Rad MRC UV laser-scanning confocal microscopy, Bio-Rad, Richmond, California) was performed on ethanol-fixed frozen brain sections (8 μM) obtained from MHV-infected $NOS2^{+/+}$ and $NOS2^{-/-}$ mice at day 7 p.i. For visualization of NeuN-positive cells, a TRITC-labeled secondary antibody was used (1:50, Sigma, St. Louis, Missouri). TUNEL-positive cells were detected through use of FITC-conjugated enzyme TdT (Boehringer Mannheim). Such analysis revealed that >70% of TUNEL-positive cells present in $NOS2^{+/+}$ mice were neurons suggesting that these cells were apoptotic (Figure 4, Table 2). In contrast, only limited numbers of TUNEL-positive cells were seen within the brains of $NOS2^{-/-}$ mice and less than 5% were neurons (Figure 4, Table 2). Sham mice of both groups showed limited TUNEL-staining. Neither macrophage/microglia nor astrocytes were found to be a major source of apoptotic cells within the brains of infected mice (data not shown).

The findings presented indicate that NOS2-generated NO does not contribute to demyelination in MHV-infected mice. These data are consistent with a recent study by Wu *et al* (2000) that indicated that NOS2 is not required for demyelination to occur following MHV infection of the CNS. In addition, Oleszak *et al* (1997) have reported that NOS2-derived NO does not participate in demyelination within mice infected with Theiler's murine encephalomyelitis virus (TMEV). Therefore, the collective evidence indicates that NOS2 expression is not important in contributing to demyelination in two separate viral models of the human demyelinating disease multiple sclerosis (MS). However, the results presented are in slight contrast with a recent report that indicated that NOS2-generated NO exhibited a transient effect on leukocyte infiltration and demyelination within the CNS of MHV-infected mice that correlated with a marked decrease in expression of mRNA transcripts for the proinflammatory chemokines CCL5 and CCL2 (Lane *et al*, 1999). Collectively, these results suggested NOS2 activity was important in the induction phase of disease, in part, by regulating chemokine expression but did not substantially contribute to the chronic stage of disease. It should be emphasized that

Table 2 Co-localization of TUNEL-positive and NeuN-positive cells

Mouse	*TUNEL-positive and NeuN-positive (percent)*	n^b
$NOS2^{+/+}$ 7 days p.i.	$71.58\% \pm 3.94\%^a$	8
$NOS2^{-/-}$ 7 days p.i.	$<5^*$	6

[a]Data are presented as mean ± SEM.
[b]n = number of fields counted. Enumeration of dual-positive cells present within the DG and hippocampus was performed on brain sections from infected mice at day 7 p.i. from staining shown in Figure 4. A minimum of two mice per condition were counted. Sham brain sections showed less than 1% of the cells to be TUNEL-positive.
*$P \leq 0.001$.

these studies were performed in MHV-infected mice treated with the NOS2 inhibitor AG (Lane *et al*, 1999). We have not found any evidence of significantly altered chemokine gene expression within the CNS of MHV-infected $NOS2^{-/-}$ mice, suggesting that AG treatment may have a direct effect on chemokine transcription. In support of this is preliminary data indicating that AG treatment of cultured macrophages directly modulates chemokine gene expression (Chen and Lane, unpublished observations). Therefore, the results presented in the current study clearly indicate that NOS2-derived NO does not affect leukocyte entry into the CNS (data not shown) nor the induction of demyelination early following infection (day 12 p.i.) or at later times (day 35 p.i.) (Table 2).

Expression of NOS2 has been associated with virus-induced neuropathology in several systems including borna virus (Koprowski *et al*, 1993; Akaike *et al*, 1995), HIV (Raber *et al*, 1996), LCMV (Campbell, 1996), and HSV-1 (Meyding-Lamade *et al*, 1998). Although the mechanisms involved in NOS2-mediated pathology within these different systems are not clear, it is possible that NO exerts a cytotoxic effect on resident cells of the CNS. Our data support a role for NOS2-derived NO in contributing to virus-induced neurologic disease by triggering events that lead to neuronal apoptosis. In support of this observation is a recent study by Rose *et al* (1998) that reported a significant reduction in apoptosis within the CNS in TMEV-infected mice treated with the NOS inhibitor AG.

It is interesting to note that Wu *et al* (2000) did not report any differences in the severity of clinical disease between $NOS2^{+/+}$ and $NOS2^{-/-}$ mice following infection with MHV, which is marked contrast to the results presented in the current report. However, that study was performed with MHV strain J2-2-V1, which triggers a mild encephalomyelitis (in which the majority of mice survive) characterized by sparing of neurons and replication in glial cells. In contrast, the virus used in the present studies (MHV-V5A13.1) is capable of replicating within both neurons and glia during acute disease and results in more severe encephalomyelitis and clinical disease. Therefore, the differences between the present study and Wu *et al* (2000) may be the result of viral strain differences and tropism for neurons.

The data presented support previous studies that have clearly shown that NO is capable of inducing death in neurons (Dawson *et al*, 1994; Zhang *et al*, 1994; Keane *et al*, 1997; Melkova *et al*, 1997; Tamatani *et al*, 1998; Yuan and Yankner, 2000). NO may trigger apoptosis by a variety of mechanisms including directly damaging DNA as well as inhibiting expression of the anti-apoptotic protein Bcl-2 (Nguyen *et al*, 1992; Mebmer *et al*, 1996; Antonsson *et al*, 1997; Dimmeler and Zeiher, 1997; Keane *et al*, 1997; Tamatani *et al*, 1998; Allione *et al*, 1999; Yuan and Yankner, 2000). The mechanism(s) by which NO induces apoptosis in neurons following MHV infection are unknown and are the focus of ongoing study.

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