Neuroprotection by Selective Inhibition of Inducible Nitric Oxide Synthase after Experimental Brain Contusion

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ABSTRACT

The inflammatory response is thought to be important for secondary damage following traumatic brain injury (TBI). The inducible nitric oxide synthase (iNOS) isoform is a mediator in inflammatory reactions and may catalyze substantial synthesis of NO in the injured brain. This study was undertaken to analyze neuronal degeneration and survival, cellular apoptosis and formation of nitrotyrosine following treatment with the iNOS-inhibitor L-N-iminoethyl-lysine (L-NIL) in a model of brain contusion. A brain contusion was produced using a weight-drop device in 30 rats. The animals received treatment with L-NIL or NaCl at 15 min and 12 h after the injury and were sacrificed at 24 h or 6 days after trauma. iNOS activity was measured at 24 h post-trauma by the conversion of L-[U-14C]arginine to L-[U-14C]citrulline and immunohistochemistry for iNOS. Peroxynitrite formation was indirectly assessed by nitrotyrosine (NT) immunohistochemistry. Neuronal degeneration and survival were assessed by Fluoro-Jade (FJ) and NeuN stainings, and cellular death by TUNEL staining. iNOS activity but not iNOS immunoreactivity was significantly reduced in animals that received L-NIL. Neuronal degeneration (FJ) and NT immunoreactivity were significantly reduced at 24 h. Neuronal survival was unchanged at 24 h but increased at 6 days in L-NIL–treated animals. Cellular apoptosis of ED-1 and NeuN positive cells was significantly reduced following L-NIL treatment at 6 days after trauma. We demonstrated neuroprotection by selective inhibition of iNOS after trauma. L-NIL appeared to protect the injured brain by limiting peroxynitrite formation. Our findings support a putative harmful role of iNOS induction early after TBI.

Key words: apoptosis; iNOS; L-NIL; neuronal degeneration; nitrotyrosine; traumatic brain injury

INTRODUCTION

The inflammatory response is thought to be important for secondary damage after traumatic brain injury (TBI) (Arvin et al., 1996). TBI induces an inflammatory reaction with production of proinflammatory cytokines (Arvin et al., 1996; Holmin et al., 1997). Nitric oxide (NO) and peroxynitrite, formed by NO and superoxide anions, are produced following TBI and may induce DNA damage, mitochondrial dysfunction, apoptosis, poly(ADP-ribose)polymerase activity (PARP), and lipid peroxidation (Bolanos et al., 1997; Hall et al., 1999;
Hogg et al., 1993; Ischiropoulos et al., 1992a; Nguyen et al., 1992; Salgo et al., 1995; Szabo and Ohshima, 1997; Whalen et al., 1999). NO is produced in the brain by three different izoenzymes: the endothelial form (eNOS), the neuronal form (nNOS), and the inducible form (iNOS). eNOS and nNOS constitutively produce NO in a calcium/calmodulin-dependent manner, whereas iNOS produces NO independently of calcium and only under certain pathologic conditions (Iadecola, 1997). The proinflammatory cytokines TNF-α and IL-1β are two major inducers of iNOS mRNA transcription and act through activation of NFκB pathway (Ganster and Geller, 2000; Kleine et al., 2000). Thus, iNOS is a possible actor in inflammatory reactions and may catalyze synthesis of NO in the injured brain (Iadecola, 1997; Minc-Golomb et al., 1994). iNOS is upregulated following experimental and clinical TBI (Clark et al., 1996; Gahm et al., 2000; Petrov et al., 2000; Wada et al., 1998). Experimental studies on cerebral ischemia have indicated that NO derived from iNOS is detrimental (Hara et al., 1996; Huang et al., 1994; Iadecola et al., 1994; Iadecola et al., 1997; Iadecola et al., 1995), but there are conflicting data for TBI, supporting both a beneficial and a detrimental role for iNOS (Bayir et al., 2005; Jafarian-Tehrani et al., 2005; Lu et al., 2003b; Sinz et al., 1999; Stoffel et al., 2000; Wada et al., 1998). This study was undertaken to further analyze the role of iNOS in experimental contusion. Neuronal degeneration and survival, cellular apoptosis and formation of nitrotyrosine at 24 h and 6 days after experimental brain contusion by a weight-drop device in rats and posttraumatic treatment with the selective iNOS-inhibitor L-N-iminoethyl-lysine (L-NIL) were assessed.

**METHODS**

Thirty female Sprague-Dawley rats, weighing 250–300 g, were anesthetized by intramuscular injection of 0.2 mL of Hypnorm (10 mg/mL fluanisone and 0.315 mg/mL fentanyl citrate) and 0.2 mL Midazolam (1 mg/mL midazolam). In addition, 0.05 mL of Xylocain (5 mg/mL lidocaine) was injected subcutaneously in the sagittal midline of the skull before the skin incision was made. The rats were placed in a stereotactic frame and a craniotomy (2 mm in diameter) was drilled under microscopic guidance at a point 2 mm posterior and 2 mm right of the bregma. A small standardized parietal contusion was produced by dropping a weight onto the exposed dura as described by Feeney et al. (1981) and modified by our research group (Holmin et al., 1995). The bone (stored in saline during the procedure) was replaced, the skin was sutured and the animals were allowed to recover. Four animals were sham operated. All animals showed normal grooming and feeding behavior within 12 h after surgery. The animals were treated with the iNOS inhibitor L-NIL 5 mg/kg or NaCl 9 mg/mL, 0.3 mL i.p, 15 min and 12 h post-trauma. L-NIL is a NOS inhibitor with a higher selectivity for iNOS over eNOS and nNOS (Alderton et al., 2001). The dose of L-NIL was chosen according to a pharmacokinetic study (Zhang et al., 2004). The animals were sacrificed at 24 h (n = 20) or 6 days (n = 10) after the trauma by decapitation in anesthesia (0.2 mL of Hypnorm and 0.2 mL of Midazolam). The brains for immunohistochemical analysis (n = 20) were removed, snap frozen in isopentane, and stored in −70°C. Coronal cryosections (14 μm) were cut through the center of the traumatized area, dried at room temperature for 1–2 h, and stored at −20°C. Prior to immunohistochemical staining, the sections were dried at room temperature for 30 min, rehydrated in diluted PBS, and fixed in 4% buffered paraformaldehyde at room temperature for 10 min. Brains for citrulline assay (n = 10) were quickly removed and frozen in cold iso-pentane. A well-defined contused area was easily recognized in all rats. The contused area and adjacent normal cortex was dissected in one piece and stored in −20°C. An equally sized piece from the corresponding area in the contralateral hemisphere was obtained from each traumatized brain (n = 10) and from normal brain (n = 4) to serve as controls in the citrulline assay. The local ethics committee approved all experiments.

**Citrulline Assay**

NOS activity was measured by the conversion of 1-[U-14C]arginine to 1-[U-14C]citrulline as described (Salter et al., 1991). Briefly, the frozen tissue was homogenized in two volumes of ice-cold buffer containing 320 mM sucrose, 10 mM Hepes, 0.1 mM EGTA, 1 mM d,L-dithiothreitol, 10 μg/mL trypsin inhibitor, 10–μg/mL leupeptin, 100–μg/mL phenylmethylsulfonyl fluoride and 2–μg/mL aprotinin (adjusted to pH 7.2 at 20°C with 1 M HCl). The homogenate was centrifuged at 10,000g for 30 min at 4°C, and the supernatants were collected and stored on ice before analysis. To measure the NOS activity, 20 μL of the supernatants were added to tubes prewarmed to 37°C and containing 100 μL of buffer (50 mM potassium phosphate [pH 7.2], 50 mM L-valine, 100 μM NADPH, 1 mM L-citrulline, 20 μM L-arginine and 1-L-[U-14C]arginine [150,000 dpm], and 1.2 mM CaCl2). Duplicates of each sample were incubated for 10 min at 37°C in the presence or absence of either EGTA (2 mM) or EGTA plus N[omega]-monomethyl-L-arginine (2 mM each) to determine the levels of the calcium-independent and the calcium-dependent activities. The reaction was terminated by removal of the substrate and dilution with 1.5 mL of 1:1 (v/v) water/Dowex AF 50W-X8 (pH 7.5).
Five milliliters of water was added to the incubation mix, and 2 mL of the supernatant was removed and examined for the presence of $L-\text{[U-14C]}$citrulline by liquid scintillation counting. The level of citrulline was expressed as pmol per gram of tissue (wet weight) per minute. All measurements were done in a blinded fashion.

**Immunohistochemistry**

The sections were fixed in 4% formaldehyde, washed and incubated in 0.3% hydrogen peroxide to quench endogenous peroxidase activity. All primary and secondary antibodies were diluted in 1% and 4% bovine serum albumine (BSA), respectively. The indirect peroxidase method was used for iNOS stainings. Sections were incubated overnight with polyclonal rabbit anti-iNOS (1:800; Transduction Laboratories, Lexington, KY). Peroxidase-conjugated goat anti-rabbit Ig (Jackson ImmunoResearch Lab. Inc., West Grove, PA), dilution 1:250, was used as conjugate. Normal goat serum (NGS) was used to prevent nonspecific conjugate binding. All conjugates were absorbed with normal rat serum to minimize cross reactivity with rat Ig. After washing, the bound peroxidase was visualized by incubation for 5 min with a diaminobezidine (DAB) substrate kit (brown; SK-4100, Vector Laboratories Inc., Burlingame, CA). Sections were counterstained with Meyer’s hematoxylin (blue), dehydrated and mounted with DPX (Distrene 80, dibutyl phthalate, xylene; BDH Laboratory Supplies, Poole, UK).

For NeuN stainings, a Vectastain Elite avidin-biotinylated enzyme complex (ABC) peroxidase kit (Vector Laboratories) was used. In brief, before the sections were incubated with the polyclonal NeuN primary antibody (1:500; Chemicon International, Ternecula, CA.), blocking serum (PBS plus 0.3% Triton plus 1% BSA) was used to prevent nonspecific conjugate binding to the primary antibodies. An avidin-biotin blocking step was then performed with ABC, to prevent nonspecific conjugate binding to endogenous biotinylated proteins. Subsequently, the sections were then incubated with the primary antibody NeuN overnight at +4°C. The indirect peroxidase method was used for detection of the primary antibodies. Biotin-conjugated goat anti-rabbit immunoglobulin (dilution 1:200; Jackson ImmunoResearch Laboratories) was used as conjugate. The sections were incubated with the ABC standard kit. The bound peroxidase was detected via incubation with a DAB substrate kit. Finally, the sections were dehydrated and mounted with DPX.

Residual neurons were calculated at the final end-point 6 days after trauma. NeuN-positive cells were quantified using a Leica DFC 320 camera and the software program Adobe Photoshop 7.0. Photomicrographs (1.03 × 1.03mm; 50×) were also taken of the contralateral hemisphere, where the tissue was morphologically intact. These images were used to exclude artefactual differences of NeuN-expression due to possible tissue damage from cryosectioning. We performed regions of interest (ROI) image analysis of NeuN-positive cells in KODAK 1D Image Analysis Software. The number of detected ROI from the medial and the lateral penumbral zone were summarized as a representation of the penumbral zone. From each animal, three similarly located sections obtained at 40-μm intervals were analyzed. The number of positive cells was averaged to constitute one observation. All measurements were done in a blinded fashion.

Nitrotyrosine (NT) is a specific biomarker for peroxynitrite-induced cellular damage (Beckman et al., 1994), as nitration of the ortho position of tyrosine is a major result of the peroxynitrite attack on proteins (Ishihara et al., 1992b). NT formation was assessed at 24 h after trauma using immunohistochemistry with NT-specific antibodies (1:200; Upstate Biotechnology, Lake Placid, NY). Sections were fixed in 4% formaldehyde and stained according to the immunohistochemistry protocol from the company (Upstate Biotechnology), using the indirect peroxidase method with DAB substrate. A positive control was selected according to the manufacturer’s recommendations, whereas preincubation with 1 mM sodium nitrite, 1 mM hydrogenperoxide in 100 mM sodium acetate, pH 5.0, BSA instead of primary antibody
culation filter, 450–490 nm; suppression filter, 515–560 nm) for fluorescent microscopy (Leica DM400B™) under high power (400×) of the area illustrated in Figure 1. Four consecutive sections from each animal stained for iNOS and NT, respectively, were analyzed and averaged to constitute one observation. All measurements were done in a blinded fashion. NT staining was evaluated only in the contusion and the penumbral zone. The levels of NT staining were scored as described by Hooper et al. (2000): 0 = none; 1 = 1–10 positive discrete loci of staining in a brain coronal section; 2 = 11–50 scattered discrete loci or areas of weak staining; 3 = extensive areas of strong staining.

**Fluoro-Jade and TUNEL Stainings**

Fluoro-Jade stainings were performed to detect neuronal degeneration (Schmued et al., 1997). Prior to staining, the sections were dried at room temperature for 1 hr and fixed in 4% buffered proformaldehyde for 10 min at room temperature. After washing in PBS, the sections were rinsed in distilled water and incubated with 0.00002% Fluoro-Jade in 0.1% acetic acid for 30 min in room temperature in a Coplin jar on a shaker. After washing in distilled water, sections were dried on a hot plate (approximately 50°C) and mounted with DPX. Programmed cell death in situ was analyzed by specific labeling of nuclear DNA fragmentation (TUNEL) (Gavrieli et al., 1992). The sections were prepared as described for immunohistochemistry except that they were post-fixed in ethanol/acetic acid 2:1 for 5 min at −20°C prior to staining. The TUNEL reaction was carried out with the In Situ Cell Death Detection Kit–Fluorescein (Boehringer Mannheim, Bromma, Sweden) at 37°C for 30 min. The slides were washed in PBS and mounted with Glycerol: PBS (2:1) and stored at +4°C. Fluorescence double labeling for TUNEL and cellular markers NeuN (neurons) and ED-1 (monocytes/macrophages) was used. The TUNEL single labeling procedure was followed, but before mounting, the sections were incubated with NGS for 30 min and primary antibodies for NeuN or ED-1 (dilution 1:4000, Serotec) were applied and incubated overnight at +4°C. After washing, an indocarbocyanine (Cy3)–conjugated donkey anti-mouse Fab2 fragments (Jackson ImmunoResearch Lab. Inc.), dilution 1:800, was applied for 1 h. Finally, the sections were washed and mounted with glycerol/PBS.

Evaluation of Fluoro-Jade and TUNEL staining was performed in a blinded fashion using a Leica DM40B™ fluorescence microscope with a Leica filter cube L4 (excitation filter, 450–490 nm; suppression filter, 515–560 nm). Cells positive for Fluoro-Jade and TUNEL, respectively, were counted under high power (400 to 1000×) in the area illustrated in Figure 1. Two consecutive sections from each animal were analyzed and averaged to constitute one observation. Similar to earlier observations following experimental TBI (Rink et al., 1995; Conti et al., 1998), TUNEL-positive cells exhibited both apoptotic and non-apoptotic morphologies. TUNEL-positive cells were further identified as apoptotic or non-apoptotic based on a presence of two or more of the classic morphologic hallmarks of apoptosis: membrane blebbing, chromatin condensation, nuclear shrinkage and cytoplasm condensation and disintegration. Apoptotic cells exhibited intense nuclear staining and breakdown of the cell surface into spherical apoptotic bodies, whereas swollen, diffusely TUNEL-positive cells without apoptotic morphology were considered to be non-apoptotic. Only TUNEL-positive cells with apoptotic morphology were counted. The proportions of neurons and macrophages among TUNEL-positive cells (with apoptotic morphology) were estimated under high power (400×). The numbers of TUNEL-positive that co-expressed NeuN or ED-1 of 100 TUNEL-positive cells were counted in a randomly chosen area that contained apoptotic TUNEL-positive cells. Double-labelled slides were evaluated using a Leica DMRB™ fluorescence microscope with a Leica filter cube L4 (excitation filter, 450–490 nm; suppression filter, 515–560 nm) for FITC labelling and with Leica filter cube N2.1 (excitation filter, 515–560 nm; suppression filter edge wavelength, 590 nm) for Cy 3 labeling.

**Statistical Analysis**

Data are presented as means ± standard deviation (SD). An unpaired t-test with Welch correction was used for evaluation of calcium-dependent and calcium-independent NOS activity and morphological data for Fluoro-Jade, NeuN, TUNEL, NT, and iNOS stainings. The Gaussian distribution of the data was tested by the method of Kolmogorov and Smirnov. p < 0.05 was considered significant. All statistical analyses were performed with the software GraphPad Instat (GraphPad Software, San Diego, CA).

**RESULTS**

**NOS Activity and iNOS Expression**

The level of citrulline converted from l-arginine by calcium-independent NOS (iNOS) in the ipsilateral hemisphere (including the contusional area) was reduced by 59% in animals treated with L-NIL compared to controls (<0.05; Fig. 2A). No difference was detected in the con-
tralateral hemisphere (Fig. 2A). The level of citrulline converted from L-arginine by calcium-dependent NOS (eNOS + nNOS) was not affected by L-NIL treatment (ipsilateral: 719.6 ± 377.5 and 609 ± 219.9, respectively; contralateral: 1050.6 ± 669.3 and 1343.4 ± 487.2, respectively). The average numbers of iNOS-positive cells in coronal brain sections through the contusion were not significantly different between L-NIL–treated animals and controls at 24 h (Fig. 2B). At 6 days after trauma, the numbers of iNOS-positive cells were small, and significant differences were not detected (data not shown).

**Nitrotyrosine**

The level of nitrotyrosine (NT) immunoreactivity at 24 h after trauma was significantly reduced in L-NIL–treated animals compared to controls (Fig. 3). Cells with NT immunoreactivity showed morphologies of macrophages, polymorphonuclear cells, astrocytes, or neurons.

**Neuronal Degeneration (Fluoro-Jade), Survival (NeuN), and Cellular Apoptosis (TUNEL)**

At 24 h, the number of Fluoro-Jade (FJ)–positive cells in the traumatized area was significantly lower in L-NIL–treated animals compared to controls (Fig. 4A,C–D), whereas no differences were detected in the numbers of TUNEL-positive cells with apoptotic morphology or residual NeuN-positive cells (Fig. 4A,B). At 6 days, the number of TUNEL-positive cells with apoptotic morphology was significantly lower in L-NIL–treated animals compared to controls, whereas no significant difference were seen for FJ positivity (Fig. 5A). A significant increase of NeuN-positive cells was seen following L-NIL treatment compared to controls at 6 days post-trauma (Fig. 5B–D). No significant differences were detected in the average numbers of NeuN-positive cells between the contralateral hemisphere of controls, of L-NIL treated animals, or between controls and L-NIL treated animals (data not shown). In our previous studies, we have not seen any morphological damage on the cortical neurons in the contralateral hemisphere. In this study, we therefore assumed that a possible detectable significant difference in the number of cortical neurons in the contralateral hemisphere was more likely to be a methodological error than a treatment re-
lated effect. Bilateral morphological changes have recently been demonstrated in a similar TBI model (Clausen et al., 2005)—however, only in the hippocampus. The proportions of TUNEL-positive apoptotic cells that co-expressed NeuN at 24 h and NeuN or ED-1 at 6 days are illustrated in Table 1; no significant differences were detected. In all groups, approximately 10–15% of all TUNEL-positive cells displayed a non-apoptotic mor-

FIG. 4. (A) Neuronal degeneration and cellular apoptosis 24 h after trauma. The number of degenerating neurons (Fluoro-Jade–positive cells) was significantly reduced \( p = 0.0107 \), whereas cellular apoptosis (TUNEL-positive + morphological criteria) was unchanged following L-NIL treatment \( p = 0.9746 \); unpaired \( t \)-test with Welch correction). \(* p < 0.05\). (B) Neuronal survival at 24 h after trauma. The number of residual neurons (NeuN-positive cells) in the perilesional cortex was not affected by L-NIL treatment \( p = 0.8472 \); unpaired \( t \)-test with Welch correction). (C,D) Illustrative photomicrographs in the perilesional cortex next to the contusion (penumbral zone), showing reduced neuronal degeneration (Fluoro-Jade–positive cells) in animals treated with L-NIL (D) compared to NaCl (C). Scale bar = 100 μm.
most non-apoptotic TUNEL-positive cells were detected centrally in the contusions.

**DISCUSSION**

We have demonstrated neuroprotection by treatment with the iNOS-inhibitor L-NIL in a model of brain contusion. Neuronal degeneration was decreased at 24 h; cellular apoptosis was decreased and neuronal survival increased at 6 days post-trauma. The inhibition of iNOS by L-NIL was confirmed by the reduced enzymatic activity seen for calcium-independent NOS in the injured area. The immunoreactivity for nitrotyrosine was significantly reduced in L-NIL–treated animals, which suggested that

**FIG. 5.** (A) Neuronal degeneration and cellular apoptosis 6 days after trauma. The number of degenerating neurons (Fluoro-Jade–positive cells) was not significantly affected (p = 0.0822), whereas a significant reduction of cellular apoptosis (TUNEL-positive cells with apoptotic morphology) was seen following L-NIL treatment compared to controls (p = 0.0450; unpaired t-test with Welch correction). *p < 0.05. (B) Neuronal survival at 6 days after trauma. The number of residual neurons (NeuN-positive cells) in the perilesional cortex was significantly higher in animals that received L-NIL (p = 0.0036; unpaired t-test with Welch correction). **p < 0.01. (C,D) Illustrative photomicrographs in the perilesional cortex next to the contusion showing increased density of neurons (cells positive for NeuN) in animals treated with L-NIL (D) compared to NaCl (C). Morphologically, the cortical cavitations did not obviously differ in size between the treatment groups but neuronal neuropil was better preserved in L-NIL treated animals (D) than in controls (C). Scale bar = 200 μm.
one of the mechanisms for neuroprotection could be the limitation of peroxynitrite formation from iNOS-derived NO production.

**NO-Mediated Toxicity**

NO may contribute to neuronal damage in different ways following TBI. High levels of NO can damage DNA directly (Nguyen et al., 1992), inhibit mitochondrial respiration (Bolanos et al., 1997; Dawson et al., 1992), or induce neuronal apoptosis via activation or upregulation of the pro-apoptotic mediators p53, Bax, and caspases (Brune et al., 1998). NO also reacts with superoxide to form the powerful neurotoxic oxidant, peroxynitrite, which causes protein tyrosine nitration, lipid peroxidation (Hogg et al., 1993; Ischiropoulos et al., 1992a), DNA damage through activation of poly(ADP-ribose)polymerase (PARP), leading to energy depletion in the cell (Salgo et al., 1995; Szabo and Ohshima, 1997), and mitochondrial dysfunction (Radi et al., 1994; Yamakura et al., 1998). Peroxynitrite is detrimental to the brain after TBI (Hall et al., 1999; Whalen et al., 1999); peroxynitrite appears to be the main mediator for NO and superoxide-produced neuronal injury in this model (Gahm et al., 2000).

In this model of brain contusion, iNOS is upregulated early, with a peak at 24 h post-trauma, and expressed mainly in macrophages, microglia, and infiltrating neutrophiles (Gahm et al., 2000). Reactive oxygen species (ROS), for example, superoxide, are generated early following TBI (Demopoulos et al., 1980; Hall, 1989)—as a result of, for example, increased intracellular calcium levels and interstitial glutamate that affect the protein expression (Nilsson et al., 1990, 1993; Schulz et al., 1995), or later from activated neutrophiles (Matsuo et al., 1995). It is known that the inflammatory reaction following TBI includes migration and infiltration of activated neutrophiles and monocytes from the blood stream into the brain parenchyma (Arvin et al., 1996) in this model of brain contusion (Holmin et al., 1995). Taken together, there are several potential sources of the two substrates for peroxynitrite following TBI: first during the acute, early ischemic phase and subsequently during the delayed inflammatory reaction.

We used immunoreactivity for nitrotyrosine (NT) as a marker for peroxynitrite formation. Peroxynitrite has a short half-life and its formation cannot be determined directly, but its product NT is detectable and provides evidence of peroxynitrite formation (Beckman et al., 1994). NT was previously used as a marker for peroxynitrite formation in experimental cerebral ischemia, TBI and following carbon monoxide poisoning (Coeroli et al., 1998; Ischiropoulos et al., 1996; Mesenge et al., 1998).

We demonstrated a significant reduction in the number of NT-immunoreactive cells after inhibition of iNOS-derived NO by L-NIL treatment. A decreased tyrosine nitration has been shown in experimental TBI following treatment with non-selective NOS inhibitors (Mesenge et al., 1998; Gahm et al., 2005), but the present study is, to our knowledge, the first to demonstrate reduced NT immunoreactivity following treatment with a selective iNOS inhibitor following experimental brain contusion. As expected, the iNOS immunoreactivity was unchanged by L-NIL, since L-NIL inactivates the enzyme by altering the functionality of its active site of iNOS (Bryk and Wolff, 1998) rather than affecting the iNOS protein concentration.

**Neuronal Degeneration**

At 24 h post-trauma, the number of degenerating neurons was decreased by L-NIL treatment, which is in agreement with recent findings with a lateral fluid percussion (LFP)–TBI (Lu et al., 2003b). Neuronal apoptosis (TUNEL-positive apoptotic cells co-labeled with NeuN) was not significantly different between the groups, which indicated that L-NIL treatment mainly affected neuronal necrosis at 24 h. An explanation of this finding might be that inhibition of iNOS by L-NIL might have limited peroxynitrite-induced activation of PARP, and thus reduced neuronal necrosis in this TBI model. Apoptosis requires energy and intracellular energy levels may regulate whether a cell undergoes necrosis or apoptosis; that is, energy depletion results in a necrotic

<table>
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<tr>
<th>Treatment/cellular marker</th>
<th>24 h, NeuN</th>
<th>6 days</th>
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<tr>
<td>L-NIL</td>
<td>48.2 ± 5.8</td>
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<td>NaCl</td>
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<td>8.9 ± 12.4</td>
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Data are presented as number of co-labeled cells of 100 TUNEL-positive cells.
rather than apoptotic cell death (Eguchi et al., 1997). The numbers of neurons were similar at 24 h in L-NIL-treated animals and controls, which indicated that iNOS-derived NO affected the degenerative processes in neurons still expressing NeuN at this time point. In contrast, neurons positive for Fluoro-Jade was not significantly different between treated animals and controls at 6 days post-trauma, whereas the numbers of surviving neurons was significantly increased following L-NIL treatment. This indicated that the beneficial effect seen on neuronal degeneration, following inhibition of iNOS activity by L-NIL, administrated 15 min and 12 h after the injury, was an early event, which mainly attenuated neuronal loss during the first 6 days. The decreased neuronal degeneration seen at 24 h but not at 6 days, together with the reversed pattern seen for neuronal survival (increased at 6 days but not at 24 h) is possible, since neuronal degeneration in this model is dynamic with most neurons dying early (Gahm et al., 2005). The dying neurons subsequently leave the pool of surviving neurons. The loss of neurons from this pool has not provided a significant finding at 24 h, while the pools of surviving neurons are significantly different at 6 days, when neuronal degeneration is already decreased. Total cellular apoptosis among all cell types (TUNEL + morphological criteria) was unchanged at 24 h, but significantly decreased at 6 days, which indicated that iNOS-derived NO affected cellular apoptosis somewhat later than trauma for neuronal degeneration. The relative proportion of apoptotic cells that coexpressed ED-1 or NeuN at 6 days was equal in L-NIL and control group, which indicated that L-NIL treatment reduced apoptosis also in macrophages, which is in agreement with recent findings (Lu et al., 2003a,b). This delayed effect of L-NIL treatment seen on cellular apoptosis correlates in time with the maximal intracerebral inflammatory response (Holmin et al., 1995). In agreement, others have found that NO is linked also to a decrease of brain macrophages by apoptosis (Lu et al., 2003a), since the inflammatory cells are a major source of iNOS in this model of TBI (Gahm et al., 2000).

Experimental studies of iNOS inhibition following TBI have been contradictory, showing both deleterious and protective effects of iNOS (Bayir et al., 2005; Jafarian-Tehrani et al., 2005; Lu et al., 2003b; Sinz et al., 1999; Stoffel et al., 2000; Wada et al., 1998).

In agreement with previous reports on treatment with the iNOS inhibitors aminoguanidine (AG) (Gorlach et al., 2000; Lu et al., 2003b; Stoffel et al., 2000; Wada et al., 1998) and 1400W (Jafarian-Tehrani et al., 2005), we found a neuroprotective effect by iNOS-inhibition early post-trauma. We also found a reduction of neuronal damage at 6 days following early iNOS-inhibition. Sinz et al. (1999) reported an impaired functional outcome following sustained iNOS inhibition after TBI and in iNOS knockout mice 3 weeks after injury, which indicated that long-term inhibition of iNOS-activity in the brain was detrimental. The principle of time-dependent differential CNS effects of NOS inhibition has also been demonstrated in CNS ischemia (Iadecola, 1997). Taken together, experimental TBI findings suggest a presumed detrimental effect of iNOS soon after TBI, but a beneficial effect over a prolonged period. Therapeutic iNOS inhibition should probably cover only the first days following TBI. In this TBI model, iNOS immunoreactivity is maximal during the first 24 h after trauma; hence, treatment would be beneficial at this time.

In conclusion, we demonstrated neuroprotection at 24 h and 6 days after trauma by posttraumatic treatment with the iNOS-inhibitor L-NIL in experimental brain contusion. L-NIL limited NO and peroxynitrite formation, which may be its mechanism of action. Our findings supported a harmful role of iNOS early after experimental contusion.

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NEURONAL PROTECTION OF INDUCIBLE NITRIC OXIDE SYNTHASE AFTER TBI


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