

Astroglial Nitration after Postnatal Excitotoxic Damage: Correlation with Nitric Oxide Sources, Cytoskeletal, Apoptotic and Antioxidant Proteins

LAIA ACARIN,¹ HUGO PELUFFO,¹ LUIS BARBEITO,² BERNARDO CASTELLANO,¹
and BERTA GONZÁLEZ¹

ABSTRACT

Oxygen free radicals and nitric oxide (NO) participate in the pathogenesis of acute central nervous system (CNS) injury by forming peroxynitrite, which promotes oxidative damage and tyrosine nitration. Neuronal nitration is associated with cell death, but little is known of the characteristics and cell fate of nitrated astrocytes. In this study, we have used a postnatal excitotoxic lesion model (intracortical NMDA injection) and our aims were (i) to evaluate the temporal and spatial pattern of astroglial nitration in correlation with the neuropathological process and the sources of NO; and (ii) to establish, if any, the correlation among astrocyte nitration and other events such as expression of cytoskeletal proteins, antioxidant enzymes, and cell death markers to cope with nitration and/or undergo cell death. Our results show that after postnatal excitotoxic damage two distinct waves of nitration were observed in relation to astrocytes. At 24 h post-lesion, early-nitrated astrocytes were found within the neurodegenerating area, coinciding with the time of maximal cell death. These early-nitrated astrocytes are highly ramified protoplasmic cells, showing diffuse glial fibrillary acidic protein (GFAP) content and expressing inducible NOS. At later time-points, when astrogliosis is morphologically evident, nitrated hypertrophied reactive astrocytes are observed in the penumbra and the neurodegenerated area, displaying increased expression of GFAP and vimentin cytoskeletal proteins and of metallothionein I–II and Cu/Zn superoxide dismutase antioxidant proteins. Moreover, despite revealing activated caspase-3, they do not show TUNEL labeling. In summary, we show that nitrated astrocytes *in vivo* constitute a subpopulation of highly reactive astrocytes which display high resistance towards oxidative stress induced cell death.

Key words: caspase-3; Cu/Zn SOD; metallothionein; nitrotyrosine; oxidative stress; TUNEL; vimentin

INTRODUCTION

THERE IS INCREASING EVIDENCE that oxygen free radicals and nitric oxide (NO) play an important role in the pathogenesis of acute central nervous system (CNS)

injury. The main neurotoxic properties of these elements involve the reaction of NO with superoxide anion, leading to the formation of the potent oxidant peroxynitrite (ONOO⁻) (Beckman et al., 1990; Radi et al., 1991) which promotes oxidative damage, inducing lipid perox-

¹Unit of Histology, School of Medicine, Department of Cell Biology, Physiology and Immunology, Institute of Neurosciences, Universitat Autònoma Barcelona, Bellaterra, Spain.

²Cellular and Molecular Neurobiology Division, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay.

idation, mitochondrial dysfunction, cell energy depletion, DNA damage and protein modification by the nitration of tyrosine residues (Beckman et al., 1992).

By using the detection of nitrotyrosine as a footprint for the demonstration of peroxynitrite formation, in the last few years different studies have shown the presence of tyrosine nitration under several neuropathological conditions, including ischemia (Tanaka et al., 1997; Coeroli et al., 1998; Hirabayashi et al., 2000), traumatic injury (Bidmon et al., 1998; Scott et al., 1999; Liu et al., 2000), multiple sclerosis (Oleszak et al., 1998), Alzheimer's disease (Smith et al., 1997) and amyotrophic lateral sclerosis (ALS) (Abe et al., 1995). In all these CNS pathologies, peroxynitrite is thought to play a key role in neuronal oxidative damage and cell death, as neuronal cell nitration by peroxynitrite formation causes cell dysfunction, mitochondrial damage and apoptosis (Bonfoco et al., 1995; Estévez et al., 1998; Ischiropoulos and Beckman, 2003).

Neuronal cell death is always accompanied by reactive astrogliosis, characterized by astroglial cell hypertrophy, cytoskeletal changes and production of both neurotrophic and cytotoxic molecules (Ridet et al., 1997). In this sense, reactive astroglial cells have been shown to express the inducible form of nitric oxide synthase (iNOS) and become nitrated in different neuropathological conditions (Endoh et al., 1994; Wallace and Bisland, 1994; Schmidt et al., 1995; Loihl and Murphy, 1998; Almer et al., 1999), pointing to reactive astrocytes as possible producers of detrimental peroxynitrite. In agreement, *in vitro* evidence has demonstrated that astrocytes are more resistant to peroxynitrite-induced cell damage (Bolaños et al., 1995; Cassina et al., 2002) and that this reactive species induces phenotypical changes in cultured astrocytes including up-regulation of glial fibrillary acidic protein (GFAP), transition towards a fibrous morphology, expression of iNOS and inhibition of high-affinity glutamate transporters, causing cytotoxicity for cocultured neurons (Trotti et al., 1996; Cassina et al., 2002). However, the characteristics and cell fate of nitrated peroxynitrite-exposed astrocytes after *in vivo* damage are unknown.

In this study we have used a well-characterized *in vivo* postnatal excitotoxic lesion model (Acarin et al., 1999a,b, 2000, 2002) to evaluate the temporal and spatial pattern of astroglial nitration in correlation with the neuropathological process and the sources of NO. As nitration is commonly found in cytoskeletal proteins, we have analyzed the correlation of major astroglial cytoskeletal proteins with nitration. Furthermore, we have analyzed whether nitrated astrocytes increase expression of antioxidant mechanisms to cope with nitration and/or undergo cell death.

MATERIALS AND METHODS

Excitotoxic Lesions

Nine-day-old Long-Evans black-hooded rat pups of both sexes were placed in a stereotaxic frame adapted for newborns (Kopf) under isoflurane anaesthesia. The skull was opened using a surgical blade, and 0.15 μ L of saline solution (0.9% NaCl, pH 7.4) containing 37 nmols of *N*-methyl-D-aspartate (NMDA) (Sigma, M-3262, Germany) were injected into the right sensorimotor cortex using a 0.5- μ L Hamilton microsyringe. Control animals received an injection of 0.15- μ L of the vehicle saline solution. After suture, pups were placed in a thermal pad and maintained at normothermia for 2 h before being returned to their mothers. Experimental animal work was conducted according to Spanish regulations, in agreement with European Union directives. This experimental procedure was approved by the ethical commission of the Autonomous University of Barcelona. All efforts were made to minimize animal suffering in every step.

Immunocytochemical Study of Nitrotyrosine

At 2, 4 and 10 h, and 1, 3, 5 and 7 days after NMDA or saline injection, rats were anesthetized by ether inhalation and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). A minimum of four NMDA-injected animals and two saline-injected controls were used for each survival time. Brains were postfixed in the same fixative for 2 h and sunk in a 30% sucrose solution before being frozen with dry CO₂. Coronal sections (30- μ m-thick) were obtained using a Leitz cryostat.

After endogenous peroxidase blocking with 2% H₂O₂ in 70% methanol, free-floating parallel sections were treated with 10% fetal calf serum in tris buffered saline (TBS) + 1% triton X-100 for 30 min, and incubated overnight at 4°C with either rabbit anti-nitrotyrosine polyclonal antibody (06-284, Upstate Biotechnology, Lake Placid, NY) (1:100) or rabbit anti-nitrotyrosine polyclonal antibody (4709, kindly provided by A. Estévez and J.S. Beckman). Afterwards, sections were rinsed and incubated for 1 h at room temperature with biotinylated anti-rabbit secondary antibody (RPN-1004, Amersham Pharmacia Biotech, England) (1:200). After the incubation, sections were rinsed in buffer and immersed in avidin-peroxidase (P-0364, Dakopatts, Denmark) (1:400) for another hour at room temperature. Finally, the peroxidase reaction product was visualized in 100 mL of tris buffer containing 50 mg of 3'-diaminobenzidine and 33 μ L of hydrogen peroxide. As negative controls for immunohistochemistry, sections

CHARACTERIZATION OF NITRATED ASTROCYTES *IN VIVO*

were incubated in media lacking primary antibodies. For control of nitrotyrosine labeling, some sections were incubated in nitrotyrosine primary antibody solution containing free amino acid nitrotyrosine (Sigma, N-7389) at 40 mM in TBS + 10% FCS (pH 7.4).

Double Immunohistochemistry for Nitrotyrosine and NOS Isoforms

In order to correlate astroglial nitration with the sources of NO, we performed double immunocytochemical detection of nitrotyrosine and iNOS or nNOS, isoforms responsible for the production of the largest amount of NO after CNS damage. Sections were immunoreacted for nitrotyrosine as reported above, but using Cy3-conjugated anti-rabbit secondary antibody (PA-43004, Amersham Pharmacia Biotech, Buckinghamshire, UK) (1:1000). On the same sections, iNOS and nNOS immunohistochemistry was performed by using rabbit anti-iNOS (NOS-2) polyclonal antibody (AB5382, Chemicon, CA) (1:6000) or rabbit anti-nNOS (NOS-1) polyclonal antibody (N53130, Transduction Laboratories, Lexington, KY) (1:3000) and Cy2-conjugated anti-rabbit secondary antibody (PA-42004, Amersham Pharmacia Biotech) (1:1000). Double-stained sections were analysed using a LEICA TCS 4D confocal microscope.

Double Immunohistochemistry for Nitrotyrosine and Cell Markers/Cytoskeletal Proteins

We used double-staining techniques for the identification of nitrotyrosine-positive cells by using specific cellular markers. Sections were immunoreacted for nitrotyrosine as reported above, but using Cy3-conjugated anti-rabbit secondary antibody (PA-43004, Amersham Pharmacia Biotech) (1:1000). Sections were then further processed by using one of the following primary antibodies: (i) polyclonal anti-GFAP (Z-0334, Dakopatts, Glostrup, Denmark) (1:1000) and (ii) monoclonal anti-vimentin (M725, Dakopatts, Denmark) (1:1000) for astroglial labeling; (iii) monoclonal anti-NeuN (MAB377, Chemicon, CA) (1:1000) for neuronal labeling; (iv) polyclonal anti-myeloperoxidase (MPO) (A0398, Dakopatts, Denmark) (1:400) for labeling of neutrophils. Afterwards, Cy2-conjugated anti-rabbit secondary antibody (PA-42004, Amersham Pharmacia Biotech) (1:1000), or Cy2-conjugated anti-mouse secondary antibody (PA-42002, Amersham Pharmacia Biotech) (1:1000) were used to visualize labeling.

Moreover, additional nitrotyrosine-immunoreacted sections were processed for double staining with tomato lectin histochemistry for the demonstration of microglial cells. Sections were incubated with the biotinylated lectin

from *Lycopersicon esculentum* (tomato) (Sigma, L-9389, Germany) diluted to 6 $\mu\text{g}/\text{mL}$ and with Cy2-conjugated streptavidin (PA-42000, Amersham Pharmacia Biotech) (1:1000). Double-stained sections were analyzed using a LEICA TCS 4D confocal microscope.

Double Immunohistochemistry for Nitrotyrosine and Antioxidant or Apoptotic Markers

To evaluate whether additional antioxidant mechanisms were found in nitrated astrocytes, we correlated nitrotyrosine immunolabeling with the expression of the antioxidant proteins heat shock protein 27kDa (HSP27), metallothionein I–II and Cu/Zn superoxide dismutase (Cu/Zn SOD) by performing double immunohistochemistry using the following primary antibodies: (i) polyclonal anti-HSP27 (SPA-801, Stressgene, Victoria, Canada; 1:2500); (ii) monoclonal anti-metallothionein I–II (kindly provided by J. Hidalgo, Autonomous University of Barcelona; 1:200); (iii) polyclonal anti Cu/Zn SOD (574597, Calbiochem, Darmstadt, Germany; 1:500).

Finally, we evaluated the activation of apoptotic or anti-apoptotic cell death markers in nitrated astrocytes, by analyzing the double immunolabeling for nitrotyrosine and the pro-apoptotic protein active caspase-3 using polyclonal anti-cleaved caspase-3 (9661, Cell Signaling Technology, Beverly, MA; 1:200); and the anti-apoptotic protein bcl-2 using monoclonal anti-bcl-2 (M0887, Dakopatts, Denmark; 1:100).

In addition, double labeling with nitrotyrosine and terminal dUTP nick end labeling (TUNEL) staining was also performed. Tissue sections previously processed for nitrotyrosine Cy3-immunolabeling were rinsed in Tris buffer (10 mM, pH 8) and EDTA (5 mM) and then incubated in the same buffer plus Proteinase K (20 $\mu\text{g}/\text{mL}$) for 15 min at room temperature. After several washes with EDTA (5 mM), sections were incubated for 10 min in TdT buffer (Tris 30 mM, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.7). Sections were then incubated in TdT buffer plus 0.161 Units/ μL TdT enzyme (terminal transferase, 3333566 Roche, Mannheim, Germany) and 0.0161 nmol/ μL of biotin-16-dUTP (1093070, Roche, Mannheim, Germany) for 30 min at 37°C. The reaction was stopped by washing the sections in citrate buffer (300 mM sodium chloride, 30 mM sodium citrate, 5 mM EDTA). After several washes with TBS, sections were incubated with Cy2-conjugated streptavidin (PA-42000, Amersham Pharmacia Biotech; 1:1000).

Selected sections were incubated for 5 min with a 0.00125 $\mu\text{g}/\text{mL}$ solution of 4,6-diamino-2-phenylindole (DAPI) in TBS. Double-stained sections were analyzed using a LEICA TCS 4D confocal microscope.

RESULTS

Injection of NMDA into the right sensorimotor neocortex of 9-day-old rats caused a lesion involving neuronal loss and glial response across the entire thickness of the cortex and the dorsal striatum at the level of the injection site, as we have previously described in detail (Acarin et al., 1999a,b, 2000). However, injection of saline solution caused no neuronal degeneration and only a very focal and transient glial response up to 3 days post-injection restricted to the needle track.

In regards to nitrotyrosine immunohistochemistry, results have been summarized in Figure 1. As similar results were obtained with the two different nitrotyrosine antibodies used, only results using the antibody from Upstate are shown in the figures. In saline-injected controls, only a faint nitrotyrosine labeling was observed in some neuronal cells of neocortical layers, thalamus and hippocampus, both in the ipsilateral and contralateral brain hemispheres (Fig. 1). Moreover, very faint nitrotyrosine-positive astroglial cells were observed in the cingulum of the corpus callosum (Fig. 2B). This constitutive nitrotyrosine labeling was markedly reduced in saline-injected control animals from 5 days post-injection (postnatal day 14).

In contrast, in lesioned animals, nitrotyrosine immunoreactivity was found in some neuronal cells and infiltrated neutrophils besides astrocytes, the main scope of the study (results are summarized in Fig. 1). Noteworthy, microglia/macrophages were never positive for nitrotyrosine at any of the survival times studied.

Nitrotyrosine in Astrocytes: Time course, Distribution and Characterization

According to the time of appearance, specific location, morphology and immunohistochemical labeling for NOS, antioxidant and apoptotic proteins, we distinguish two waves of nitrated astrocytes (Fig. 1 and Table 1): (i) early-activated highly ramified protoplasmic astrocytes, and (ii) late-activated reactive hypertrophied astrocytes.

Early-nitrated highly ramified protoplasmic astrocytes

showed low and diffuse GFAP content and no signs of cell hypertrophy (Figs. 2A, 3A), resembling the early activated astroglial phenotype previously described by Raivich et al. (1999) in the damaged gray matter. These highly ramified protoplasmic activated astrocytes were only observed at 24 h post-lesion evenly distributed

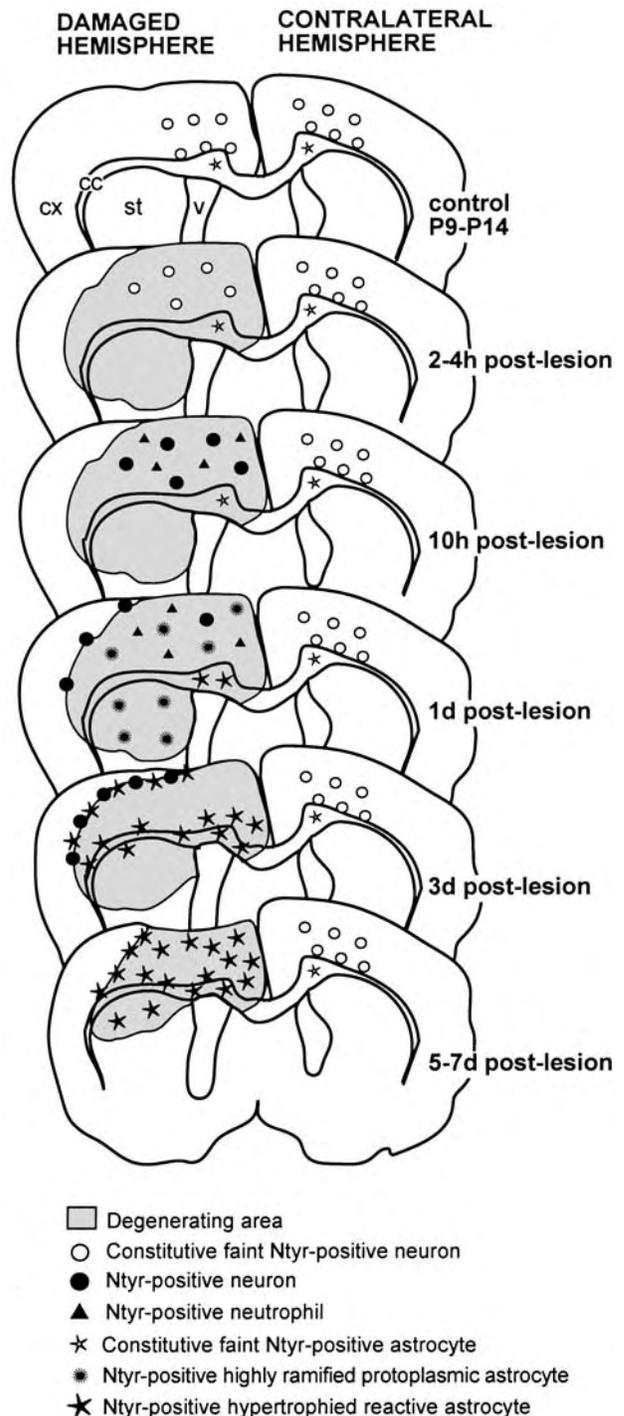


FIG. 1. Temporal and spatial distribution of nitrotyrosine-positive cells in the normal postnatal brain and at different survival times following a cortical excitotoxic lesion. The neurodegenerating area is shaded in the ipsilateral hemisphere. Nitrotyrosine-positive cells are identified as neurons (circles), neutrophils (triangles), and two morphologically distinct astrocytes (asterisks and stars). Nitrated astrocytes show a highly ramified protoplasmic morphology at 24 h post-lesion (asterisks) and are hypertrophied reactive cells at longer survival times (stars). Cx, cortex; cc, corpus callosum; st, striatum; v, ventricle.

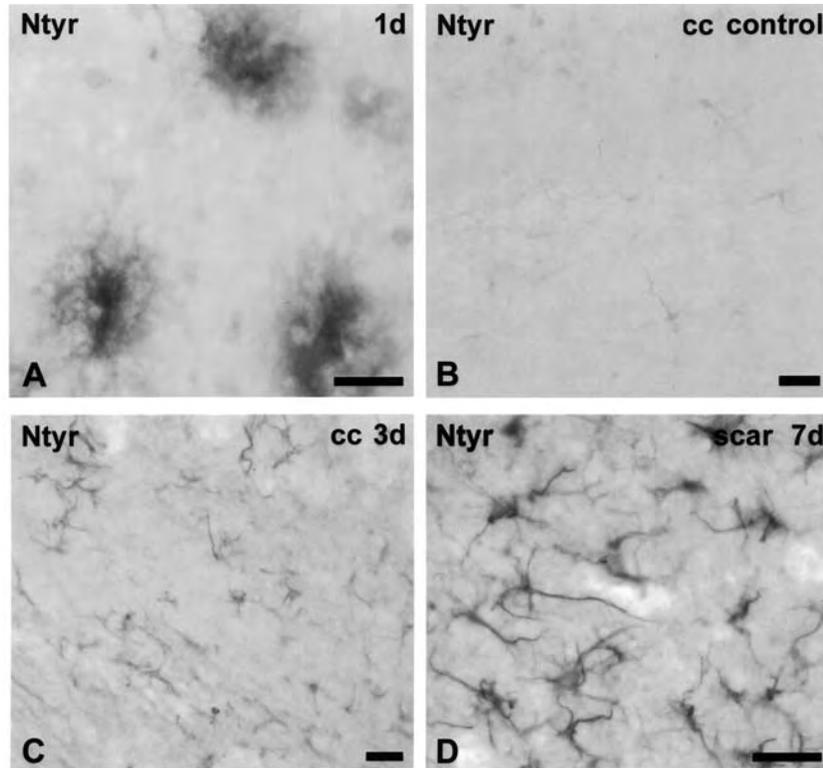


FIG. 2. Distribution of nitrotyrosine-positive (Ntyr) astrocytes. Early-nitrated astrocytes are observed at 1 day post-lesion (A) as highly ramified protoplasmic cells. In comparison to control animals (B), at 3 days post-lesion, nitrated reactive astrocytes are found in the corpus callosum (cc) (C). At 5–7 days post-lesion, nitrated highly reactive hypertrophied astrocytes cover the neurodegenerating area and form the glial scar (D). Bar = 30 μm .

throughout the neurodegenerating areas in the cortex and striatum.

Late-nitrated reactive astrocytes were observed in the, 3 days post-lesion. These cells were characteristically hypertrophied and showed increased GFAP labeling with defined and thick GFAP-positive filament bundles (Figs. 2D, 3E). At 3 days post-lesion, these nitrated reactive astrocytes were observed in the borders of the lesion, contacting penumbra neurons with their cell bodies or cytoplasmic processes (Fig. 3D), as well as in the adjacent corpus callosum (Fig. 2C), extending their projections towards the degenerating areas in the cortex. At 5–7 days post-lesion, they covered the degenerating area (Fig. 2D) and accumulated in the lateral margins of the lesion and the upper cortical layers, forming the glial scar. The phenotypical characterization of the two types of nitrated astrocytes using double labeling techniques are summarized in Table 1 and detailed below.

Nitrated Astrocytes and Sources of NO

As previously described (Acarin et al., 2002), iNOS expression in lesioned animals was strongly induced at 10–24

hours post-lesion, in highly ramified protoplasmic activated astrocytes found throughout the neurodegenerating area. Noteworthy, at 1 day post-lesion but not at earlier timepoints, iNOS expression in highly ramified protoplasmic activated astrocytes co-localized with nitrotyrosine labeling (Fig. 3B), although not all iNOS-expressing astrocytes were nitrated. In contrast, nitrated late reactive hypertrophied astrocytes seen from 3 days post-lesion, including those at the glial scar, did not display iNOS expression. No co-localization between either types of nitrated astroglia and nNOS was observed at any time, although nNOS positive neurons were always found surrounding the area occupied by nitrated astrocytes (Fig. 3C).

Nitrated Astrocytes and Cytoskeletal Proteins

Whereas nitrated early highly ramified protoplasmic astrocytes at 24 h showed low and diffuse GFAP content (Fig. 3A) and no vimentin expression, nitrated late reactive hypertrophied astrocytes seen from 3 days post-lesion showed strong GFAP labeling (Fig. 3E) and *de novo* expression of vimentin (Fig. 3F). Notably, most, but not all GFAP-positive reactive astrocytes showed nitrotyro-

TABLE 1. CHARACTERIZATION OF NITRATED ASTROCYTES FOLLOWING EXCITOTOXIC DAMAGE TO THE IMMATURE RAT BRAIN

Nitrated astrocytes	Time post-lesion	NO sources		Cytoskeletal protein		Antioxidant proteins		Apoptotic/anti-apoptotic markers		
		nNOS	iNOS	GFAP	Vimentin	Cu/Zn SOD	MT I-II	Bcl-2	Active Caspase-3	TUNEL
Highly ramified protoplasmic astrocyte	24 h	–	++	–/+ diffuse	–	–	–	–	–	–
Reactive hypertrophied astrocyte	3–7 days	–	–	++	++	+	+	–	+	–

Cu/Zn SOD, copper/zinc superoxide dismutase; GFAP, glial fibrillary acidic protein; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; TUNEL, terminal UTP nick end labeling. Grading of immunoreactivity: –, no labeling; –/+, faint labeling; +, labeling; ++, strong labeling.

sine labeling, and nitrated astrocytes were always the more hypertrophied and showing the strongest GFAP overexpression (Fig. 3E). At the cellular level, nitrotyrosine was found in thick GFAP-positive cell projections, but thinnest and distal GFAP-positive processes often lacked nitrotyrosine (Fig. 3E). In regards to vimentin, only nitrated hypertrophied astrocytes expressed vimentin, and when observed at high magnification, nitrotyrosine labeling strongly co-localized with the cellular location of this intermediate filament (Fig. 3F).

Nitrated Astrocytes and Antioxidant Proteins

Nitrated early highly ramified protoplasmic astrocytes found at 24 h post-lesion lacked expression of HSP27, metallothionein I–II, or Cu/Zn SOD (Table 1). However, the majority of nitrated late reactive hypertrophied astrocytes presented *de novo* expression of metallothionein I–II (Fig. 3G) and Cu/Zn SOD (Fig. 3H) in their soma and proximal projections. No correlation was found between nitration and HSP27 immunoreactivity (data not shown), as HSP27 induction was only observed in some nitrated astrocytes, and HSP27-positive non-nitrated astroglial cells were also found.

Nitrated Astrocytes and Apoptosis-Related Markers

Nitrated highly ramified protoplasmic astrocytes found at 24 h post-lesion neither showed activation of caspase-3 nor TUNEL staining (Table 1). In contrast, most nitrated reactive hypertrophied astrocytes found from 3 days post-lesion and, until 7 days, the last survival time analyzed, showed immunoreactivity for active caspase-3 in their nuclei (Fig. 3I,J). However, caspase-3-positive nitrated astrocytes did not show any signs of nuclear condensation using DAPI nuclear staining (Fig. 3J) and were

not positive by the TUNEL staining at any of the survival times studied (Fig. 3K,L). Finally, nitrated astrocytes did not show expression of the anti-apoptotic protein bcl-2 at any time (data not shown).

DISCUSSION

In this study, we have shown that after postnatal excitotoxic damage two distinct waves of nitrated astrocytes are observed. Around 24 h post-lesion, nitrated astrocytes are highly ramified protoplasmic cells, showing diffuse GFAP content and expressing iNOS. At later time-points, when astrogliosis is more evident, nitrated astrocytes are hypertrophied and display increased expression of the cytoskeletal proteins GFAP and vimentin, expression of the antioxidant proteins metallothionein I–II and Cu/Zn SOD, as well as activation of caspase-3 in the absence of nuclear fragmentation. These late-nitrated reactive astrocytes do not express iNOS or nNOS.

Early-Nitrated Highly Ramified Protoplasmic Astrocytes Express iNOS

Although earlier survival times of 2, 4 and 10 h post-lesion were analysed, nitration of astrocytes was not found until 24 h post-lesion, in highly ramified protoplasmic astrocytes, resembling the early-activated reactive velate astroglial phenotype that has been previously described by Raivich et al. (1999) following ischemia and trauma and recently by Campos (2003) in transgenic mice expressing *Lmo1-lacZ*. These nitrated astrocytes show iNOS expression, although not all iNOS-expressing astrocytes were nitrated. Noteworthy, iNOS expression is already seen in these types of astroglial cells at 10 h post-lesion (Acarin

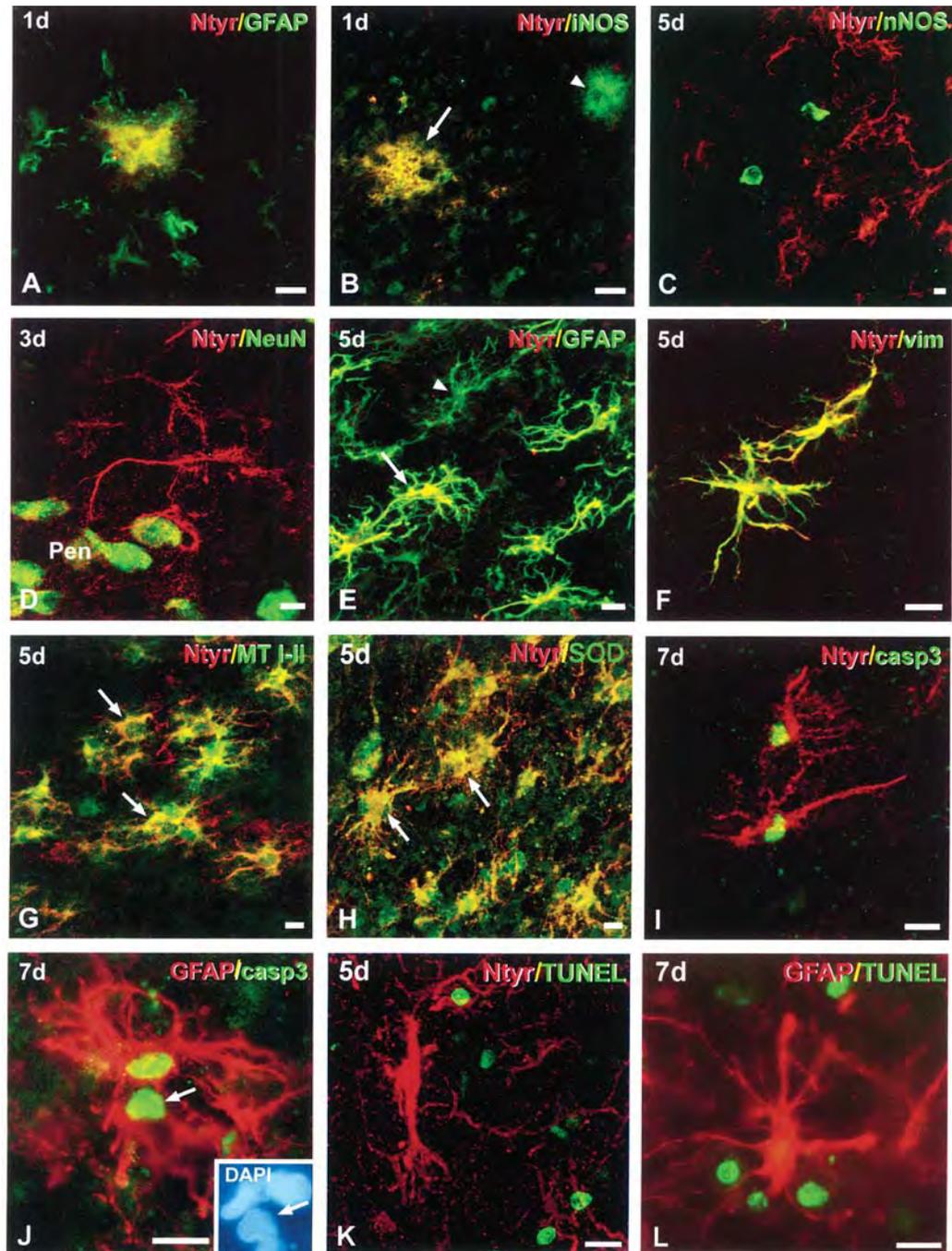


FIG. 3. Co-localization of nitrated astrocytes with NO sources and cytoskeletal, antioxidant, and apoptotic proteins. Nitrotyrosine (A–I,K) or GFAP (J,L) labeling are shown in red and either GFAP (A,E), iNOS (B), nNOS (C), NeuN (D), vimentin (vim) (F), metallothionein I-II (MT I-II) (G), Cu/Zn superoxide dismutase (SOD) (H), caspase-3 (casp3) (I–J) or TUNEL (K–L) are shown in green. Yellow shows co-localization of markers. At 24 h post-injury, nitrated hairy astrocytes show mild GFAP (A) and iNOS (arrow in B) labeling, and not all iNOS-positive astrocytes are nitrated (arrowhead in B). No co-localization between nitrated astrocytes and nNOS staining is seen at any time (C). At 3 days post-injury, some nitrated astrocytes are found surrounding NeuN-positive neurons in the penumbra region (Pen) (D). Nitrated astrocytes are reactive hypertrophied and the most strongly GFAP-positive (arrow in E), although not all GFAP-positive astrocytes are nitrated (arrowhead in E). Only nitrated astrocytes show vimentin staining (F) and nitrotyrosine labeling co-localizes with the cellular location of vimentin filaments (F). All nitrated astrocytes express MT I-II (e.g., see arrows in G) and SOD (e.g., see arrows in H). Note that mainly astrocytes express MT I-II, but SOD is expressed by nitrated astrocytes and by all neurons. Nitrated reactive astrocytes show caspase-3 positive nuclei (I and J); however, neither showed nuclear condensation by DAPI staining (arrow in inset in J) nor DNA cleavage by TUNEL labeling (K, L). Bar = 10 μ m.

et al., 2002), more than 12 h prior to nitration. In this sense, it is reasonable to think that tyrosine nitration in these astrocytes may represent peroxynitrite formed within the cell, by NO derived from endogenous iNOS in combination with superoxide anion, which is largely produced in excitotoxicity (Lafon-Cazal et al., 1993). In addition, a tyrosine nitration mechanism involving neutrophil myeloperoxidase has also been described (Eiserich et al., 1999), although no close association between neutrophils and nitrated astrocytes was found.

iNOS is the enzyme isoform associated with the production of large amounts of NO and its cytotoxic actions (Iadecola et al., 1995; Yun et al., 1996). Accordingly, iNOS is supposed to be responsible for the production of NO to form peroxynitrite after CNS damage, as nitrotyrosine formation is strongly reduced after treatment with iNOS inhibitors (Takizawa et al., 1999; Ikeno et al., 2000) and in iNOS knockout mice (Liberatore et al., 1999; Hirabayashi et al., 2000). In these models, iNOS inhibition, which may imply decreased peroxynitrite formation, is associated with neuroprotection (Iadecola et al., 1995, 1997; Lecanu et al., 1998; Liberatore et al., 1999; Tsuji et al., 2000). Therefore, in our excitotoxic model, early iNOS-expressing nitrated astrocytes, whose appearance correlates with the time of maximal neuronal death in this model (Acarin et al., 1999a), could contribute to neuronal damage, either by NO/peroxynitrite production or by toxic 3-nitrotyrosine release (Mihm et al., 2001; Cassina et al., 2002; Peluffo et al., 2004). However, it should be noted that different cell types besides astrocytes, mainly infiltrating neutrophils, become nitrated and express iNOS (Iadecola et al., 1996; Bidmon et al., 1998; Coeroli et al., 1998; Grzybicki et al., 1998; Loihl et al., 1999; Acarin et al., 2002). In this sense, the putative neurotoxic role of astroglial-derived NO and peroxynitrite, which has been demonstrated using astroglial cultures (Dawson et al., 1994; Stewart et al., 2000; Cassina et al., 2002), would be *in vivo* potentiated by neutrophils.

Nitration of Reactive Hypertrophied Astrocytes

Later on, when reactive astroglial cells cover the neurodegenerates area and form the glial scar, nitrotyrosine labeling is found in hypertrophied reactive astrocytes. These nitrated astrocytes do not show iNOS/nNOS expression, which suggests that they do not produce NO themselves, at least when nitration is occurring. Another possibility could involve the astroglial production of superoxide anion, but difficult to demonstrate *in vivo*. Reactive astrocytes in several neurodegenerative conditions are known to activate cytosolic phospholipase A2, producing arachidonic acid (Stephenson et al., 1999), an inducer of superoxide formation in astroglial cultures

(Chan et al., 1988). Superoxide anion can form peroxynitrite by combining with NO, which may reach astroglial cells by diffusion from surrounding nNOS neuronal cells, as neuronal nNOS has been previously shown to play an important role in cell nitration after NMDA-induced excitotoxicity (Ayata et al., 1997).

Nevertheless, we cannot rule out that reactive astrocytes could be nitrated by incorporating 3-nitrotyrosine from the extracellular media, released by early-nitrated astrocytes, neutrophils and neurons in the degenerating area. However, the short half-life of parenchymal nitrotyrosine after ischemia (little more than 2 h) (Takizawa et al., 1999) does not support this mechanism. In the same way, incorporation of exogenous nitrotyrosine into astroglial cells would result in the nitration of tubulin by the activity of tyrosine-tubulin ligase (Eiserich et al., 1999; Peluffo et al., 2004), and in this lesion model no nitrotyrosine-positive band around 55 KDa, the molecular weight of dissociated alpha-tubulin and other cytoskeletal proteins, is observed in western blots (H. Peluffo, unpublished observations).

In order to further characterize nitrated astrocytes and evaluate the putative nitration of other cytoskeletal proteins, we correlated nitrotyrosine labeling with the expression of the intermediate filament proteins, GFAP and vimentin. Vimentin is found in astrocytes during brain development but is lost when the brain reaches maturity and GFAP becomes the major astroglial intermediate filament protein (Dahl, 1981). However, astroglial cells show *de novo* expression of vimentin in response to several types of brain damage (Takamiya et al., 1988; Acarin et al., 1999b; Eliasson et al., 1999). Here we show that, although nitrotyrosine labeling is not found in all GFAP-overexpressing astrocytes and nitration is not observed in all GFAP-containing cell processes, nitrotyrosine co-localizes with vimentin-positive filaments. Noteworthy, all vimentin-positive astrocytes are nitrated, and all vimentin-containing cell processes show nitrotyrosine labeling. Astroglial vimentin expression has been linked to motility and proliferation (Takamiya et al., 1988; Janeczko, 1993; Lepekhn et al., 2001), intracellular transport mechanisms (Pixley and De Vellis, 1984; Hutchins and Casagrande, 1989), and as support for protein kinases (Ciesielski-Treska et al., 1995). Therefore, it is likely that proteins associated with vimentin-intermediate filaments, more than the vimentin protein itself, could become nitrated as a result of peroxynitrite formation in astrocytes.

Expression of Apoptotic and Antioxidant Proteins in Nitrated Astrocytes

Although it is known that neuronal nitration induces cellular damage and activation of apoptotic mechanisms

(Bonfoco et al., 1995; Endres et al., 1998; Estévez et al., 1998; Oka et al., 2000), the effect of nitration in astrocytes is largely unknown. Besides modifications in microtubule assembly, tyrosine nitration can disrupt the phosphorylation/dephosphorylation dynamics of tyrosine kinase residues involved in cell signalling (Kong et al., 1996; Knapp et al., 2001). In spite of this, Bolaños and coworkers have demonstrated that cultured astrocytes are more resistant to peroxynitrite-mediated mitochondrial damage due to a rise in glutathione levels and a switch towards glycolytic metabolism (Bolaños et al., 1995; Bolaños and Almeida, 1999), which may also account for the absence of astroglial death after peroxynitrite treatment *in vitro* (Cassina et al., 2002).

In this report we have observed that early-nitrated highly ramified protoplasmic astrocytes do not show activation of the apoptotic protein caspase-3, whereas late-nitrated reactive hypertrophied astrocytes do. Caspase-3 is a proteolytically activated enzyme which is considered one of the major executioners of apoptosis (Nicholson, 1999; Springer et al., 2001) and is induced by peroxynitrite in different cell types *in vitro* (Estévez et al., 1998; Lin et al., 1998; Cassina et al., 2002). Nevertheless, it should be noted that nitrated reactive astrocytes showing caspase-3-positive nuclei did not show signs of nuclear fragmentation and were TUNEL-negative, even at the last survival time studied, suggesting the existence of a time frame between caspase-3 activation and the execution of apoptotic death (Brecht et al., 2001) or pointing to an activated state of caspase-3 positive cells in the absence of cell death, as has been reported in a model of ischemic preconditioning (McLaughlin et al., 2003) and in status epilepticus (Narkilahti et al., 2003).

Finally, it is thought that one of the mechanisms by which astroglial cells compensate for oxidative stress and avoid cell death is by increasing different antioxidant mechanisms. Accordingly, in this study we have shown that, whereas no correlation between nitrated astrocytes and expression of HSP27 was observed, reactive nitrated astrocytes present *de novo* expression of Cu/Zn SOD and metallothionein I–II. Cu/Zn SOD is largely recognised as one of the main free radical scavenger in ischemia and excitotoxicity (Noack et al., 1998; Kim et al., 2000) and its over-expression provides astrocytes an increased resistance to oxidative damage (Chen et al., 2001). Metallothionein I–II is a metal-binding protein upregulated in response to stress and inflammation, playing a key role in the detoxification of heavy metals and scavenging of free radicals (Hidalgo et al., 1994). Interestingly, transgenic over-expression of metallothionein I in astrocytes and microglial cells dramatically decreases inflammation, reduces astroglial and microglial iNOS expression and diminishes lipid peroxidation and nitrotyrosine labeling,

decreasing neuronal apoptosis (Giralt et al., 2002; Penkowa et al., 2002). Accordingly, both Cu/Zn SOD and metallothionein could serve nitrated reactive hypertrophied astrocytes as an additional antioxidant mechanism to cope with oxidative stress and cell death.

In conclusion, we report that an excitotoxic lesion to the postnatal brain induces the nitration of a subpopulation of astrocytes in two waves that differ in cell characteristics and temporal location. An early wave included a rapid iNOS induction in nitrated astrocytes, coinciding with the time of maximal cell death. A second and later astrocytic nitration wave was characterized by cell hypertrophy, increased GFAP expression, and induction of vimentin and the antioxidant proteins metallothionein and Cu/Zn SOD. These nitrated astrocytes, despite displaying caspase-3 activation, seemed to be highly resistant towards oxidative stress induced cell death, probably by the overexpression of antioxidant enzymes.

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Address reprint requests to:

Laia Acarin, Ph.D.

Unitat Histologia

Facultat Medicina

Torre M5

Campus Universitat Autònoma Barcelona

Bellaterra 08193, Spain

E-mail: laia.acarin@uab.es