Theodor Petrov · Andrea B. Page · Chery R. Owen · José A. Rafols

Expression of the inducible nitric oxide synthase in distinct cellular types after traumatic brain injury: an in situ hybridization and immunocytochemical study

Abstract The Marmarou’s acceleration traumatic brain injury (TBI) model, in situ hybridization and immunocytochemistry were utilized to study the temporal expression of the inducible form of nitric oxide synthase (iNOS) mRNA and protein in different cellular compartments of the rat brain. Four hours following TBI, expression of iNOS was observed in the endothelial cells of cerebral blood vessels, macrophages and many cortical and hippocampal neurons. In the cortex labeled neuronal and non-neuronal cells were primarily found in the superficial layers. In the hippocampus the strongest neuronal labeling was observed in the CA1 and CA3 (lateral part) regions. By 24 h post TBI endothelial cells no longer expressed iNOS mRNA, and the macrophage and neuronal iNOS expression was reduced by 30–50%. The reduction was assessed by automated quantitation of the silver grains that occupy individual cellular profiles using an image analysis system. Immunocytochemistry revealed de novo iNOS synthesis in non-neuronal cells at the different time points, thus paralleling the changes in iNOS mRNA expression. In contrast, iNOS immunoreactivity in neurons was not observed before 24 h post TBI, suggesting failure of iNOS protein translation at 4 h after trauma. The results demonstrate complex spatial and temporal patterns of iNOS expression in discrete cellular populations, indicating different times of nitric oxide synthesis (and release) following TBI. Uncoupling of iNOS mRNA and protein synthesis in neurons suggests differential synthesis of nitric oxide in these cells as compared to non-neuronal cellular populations after trauma.

Key words Endothelial cells · Macrophages · Neurons · Cytoprotection · Cytotoxicity

Introduction

Traumatic brain injury (TBI) induces cellular responses that result in impaired function of the central nervous system. One of the molecules that is involved in the cascade of cellular events that leads to neuronal cell loss (or dysfunction) is nitric oxide (NO) because of its effects on apoptotic genes [41]. NO is one of the reactive oxygen species that can be highly toxic and that, in general, contribute to extensive cell damage. However, NO is also a neuromodulator [23] and we have shown that NO synthesizing neurons can be activated in response to various challenges to the central nervous system [16, 28]. Three isoforms of a synthesizing enzyme (NOS) are implicated in the synthesis of NO – nNOS (neuronal), eNOS (endothelial) and iNOS (inducible) [34]. The first two enzymes are constitutively expressed and their activation results in the generation of low amounts of NO, while iNOS is a transcriptionally regulated enzyme and its up-regulation leads to a significant increase in NO synthesis [9]. iNOS was first identified in macrophages, but was shown subsequently to be induced in other cell types including neurons [23]. iNOS can only be expressed following neuronal trauma or under other pathological conditions [35], and toxic effects of NO are attributed to the synthesis of iNOS [22].

Recent biochemical studies [36] using the impact acceleration model of Marmarou et al. [21] as well as other models [10, 42] revealed a general increase of NO/iNOS synthesis in the cortex and the diencephalon of rats subjected to TBI. For example, up-regulation of iNOS mRNA following cryogenic lesions [18] and increased protein synthesis of iNOS in the fluid percussion model [42] have been reported. However, such studies did not establish which particular cell types contributed to this increase and whether the gene that encodes for iNOS was transcriptionally activated following TBI using the model of Marmarou et al.

We undertook a combined in situ hybridization and immunocytochemical study to establish whether different cellular types in the brain contribute to up-regulation of
the gene that encodes for iNOS as well of its protein synthesis following TBI. iNOS expression at 4 and 24 h following the trauma was studied to define its precise cellular compartmentalization and also to gain insight to the source of and potential role of NO in the development of secondary cell injury after TBI.

**Materials and methods**

**Traumatic brain injury**

Male Sprague-Dawley rats (250–300 g) were initially anesthetized with 5% halothane in a chamber as previously described [13]. Briefly, animals were intubated intratracheally and supplied with a mixture of halothane (1%) and N₂O/O₂ in a ratio 2:1. The skull was exposed following a longitudinal skin incision and the rats were placed on a foam cushion under a plastic cylinder through which a weight of 450 g was dropped onto the skull from a height of 2 m [21]. A steel helmet was attached to the skull before induction of TBI to prevent skull fracture and massive bleeding, and to induce diffuse injury. After the weight struck the helmet animals were kept under 1.5% halothane anesthesia until spontaneous respiration resumed, the intubation tube was removed and the skin sutured. Recovery was tested by post injury behavioral injury assessments (PINA) which consisted in recording the length of convulsions (s) and their severity (mild, moderate, severe), as well the presence or absence of reflex responses to the hind limb, corneal and auditory meatus responses to stimulation (min), and the period needed for each animal’s recovery (min). Only rats that exhibited mild to moderate seizures, responded to reflex stimulation 10–15 min following the trauma, and recovered within 30 min were used in these experiments. Animals used for controls (n = 6 at each time point) were treated identically, except that they were not subjected to TBI.

In situ hybridization

After 4 or 24 h rats (n = 6 for each time point) were perfused with ice-cold 4% paraformaldehyde under deep pentobarbital anesthesia (0.12 ml/100 g body weight). Coronal sections, 14 μm thick, through the sensorimotor cortex and the hippocampus (-2.3 to -4.3 posterior to the bregma according to the Atlas of Paxinos and Watson [27]) were cut on a cryostat and collected on poly-l-lysine-coated slides. These brain areas were selected because of their high neuronal vulnerability following TBI injury [13, 33]. Before hybridization mounted sections were dehydrated in graded ethanol and embedded in araldite (Sigma Chemical Co., St. Louis, Mo.; 1 : 100) and 0.05% diaminobenzidine (DAB), 0.01% H₂O₂ in 0.1 M phosphate-buffered saline (PBS). Cortical tissue blocks oriented perpendicularly to the pial surface and containing all six cortical layers were treated with 2% osmium tetroxide for 60 min, dehydrated in graded ethanol and embedded in araldite as previously described [45]. Postembedding immunocytochemical methods were used in tissues prepared for future electron microscopic work as follows. Semithin (1 μm thick) sections were cut with a Reichert Ultratome and mounted on poly-l-lysine coated microscope slides as described. The sections were subsequently etched using sodium ethoxide (1 : 30 in 100% EtOH, 15 min) followed by 1 min in 100% EtOH. After rinsing with dH₂O and 5% H₂O₂, the sections were incubated for 30 min with PBS containing 0.1% Triton X, followed by normal goat serum (30 min) and, finally, incubated overnight with a rabbit polyclonal anti-iNOS antibody (Calbiochem, San Diego, Calif.; 1 : 100). Tissues were sequentially incubated for 1 h with anti-rabbit biotinylated antibody (Sigma Chemical Co., St. Louis, Mo.; 1 : 100) and avidin-biotin-peroxidase complex (ABC) reagent (Vector Laboratories, Burlingame, Calif.), and 0.05% diaminobenzidine (DAB), 0.01% H₂O₂ in 0.1 M PBS for 5 min. In addition to sections from animals not subjected to TBI, control sections from brains at 4 and 24 h post TBI were processed identically except that the primary antibody was omitted. No nonspecific labeling was observed.

**Results**

In the control (non-injured) animals the distribution of silver grains in the tissues was similar to the nonspecific labeling observed in tissues hybridized with mismatch or sense oligonucleotides, which reflects the virtual lack of iNOS expression in the intact brain. This corresponded to the lack of immunoreactivity in the tissues from non-injured animals incubated with the iNOS antibody (see below).

**Non-neuronal cells**

At 4 h following TBI endothelial cells of cerebral cortical vessels revealed dense accumulation of silver grains, in many cases delineating most of the cellular profile as well as the contour of longitudinally and cross-sectioned blood vessels (Fig. 1A). At 24 h after trauma the labeling was