Inhibition of cyclooxygenase 2 by nimesulide improves cognitive outcome more than motor outcome following diffuse traumatic brain injury in rats

Abstract Prostanoid synthesis is regulated by the enzyme cyclo-oxygenase (COX) that is present in at least two isoforms: COX-1, the constitutive form, and COX-2, the inducible form. Expression of COX-2 has recently been shown to be an important determinant of the cytotoxicity connected with inflammation following ischemic injury to the brain. The present study examines the temporal and spatial profiles of COX-2 expression following diffuse traumatic brain injury (TBI) in rats, and the effects of the COX-2 inhibitor nimesulide on cognitive and motor outcomes. Adult, male Sprague-Dawley rats were injured using the 2-meter impact acceleration model of diffuse TBI. At preselected time points after injury, animals were killed and the expression of COX-2 was measured in the hippocampus and parietal cortex by immunohistochemistry and Western blotting techniques. Effects of nimesulide (6 mg/kg daily over ten days) on cognitive and motor outcome was assessed in a separate group of animals using the Barnes circular maze and rotarod test, respectively. A highly significant up-regulation of COX-2 expression was found in the hippocampus as early as 3 h post-trauma and persisting for at least 12 days after TBI. In contrast, a slight but significant upregulation of COX-2 expression occurred in the cortex only at 3 days after trauma. Administration of the COX-2 inhibitor nimesulide resulted in a significant and substantial improvement in cognitive function compared to vehicle-treated controls, while motor deficits after injury was only improved at 24 h after injury. We conclude that COX-2 is involved in the development of functional deficits following diffuse TBI, particularly cognitive deficits, and that these can be improved by administration of COX-2 inhibitors.

Keywords Neurotrauma · COX · Cognition · Diffuse axonal injury

Introduction

There is accumulating evidence suggesting that the brain damage produced by traumatic brain injury (TBI) develops over a significant period of time after the traumatic event. Indeed, post-traumatic functional outcome is now thought to be dependent not only on primary mechanisms of tissue destruction occurring at the time of injury, but also on complex secondary injury mechanisms initiated at the time of trauma and manifesting for minutes or even days after the traumatic insult. Accordingly, the identification of these secondary injury factors may present new opportunities for therapeutic strategies targeted at the late phase of the damage (Faden 1996).

One of the secondary injury processes that may promote delayed neuronal death is post-traumatic inflammation, which has been shown to increase blood-brain barrier permeability, cerebral edema and intracranial pressure, resulting in neuronal dysfunction after TBI (DeWitt and Prough 1998; McIntosh et al. 1998). Prostaglandins are among the pivotal mediators/modulators of this inflammation and, together with thromboxanes, are part of the prostanoid family that are synthesized from arachidonic acid (5,8,11,14-eicosatetraenoic acid) via cyclooxygenase (COX, or prostaglandin H synthase), which is present in at least two isoforms (Vane et al. 1998). COX-1 is a predominantly constitutive form and is involved in cellular homeostasis, while COX-2 is an inducible isoform up-regulated by reactive oxygen species (ROS), inflammatory cytokines and mitogens (Dubois et al. 1998; Lasa et al. 2000). The fact that COX-2 has been characterized as a representative of the immediate early genes (IEGs) that can directly influence cellular function (Yamagata et al. 1993) emphasizes its
potential importance in a number of pathophysiological processes. Induction of COX-2 has profound effects on the brain via the complex effects of prostanoids, which include, among others, modulation of glutamate release (Adams et al. 1996), cerebral vasooconstriction (Brian et al. 1998), induction of ROS release (Tardieu et al. 2000), and influence on neuroendocrine function (Parsadaniantz et al. 2000). Therefore, it is possible that COX-2 upregulation in injured neurons could contribute to neuronal death following brain injury. Consistent with this, inhibition of COX-2 activity has recently been demonstrated to protect against ischemic damage (Nakayama et al. 1998; Wakita et al. 1999), kainate-induced seizures (Candelario-Jalil et al. 2000), and neurotoxicity caused by chronic neuroinflammation (Willard et al. 2000).

Although recent studies have demonstrated increased COX-2 expression in brain structures of rats following cortical contusion injury (Dash et al. 2000; Strauss et al. 2000), no studies have examined the role of COX-2 in models of traumatic brain injury that have diffuse axonal injury as a major component. This is despite the fact that diffuse axonal injury is thought to be a major component of severe clinical head injury and, in particular, the formation of the vegetative state. Accordingly, in this study we investigated whether COX-2 is expressed in the brain following diffuse TBI and, if so, whether its upregulation contributes to the secondary evolution of the damage and consequent development of cognitive and motor deficits.

**Materials and methods**

**Animal preparation**

All procedures in this study were performed in accordance with the NIH guidelines for the humane treatment of laboratory animals (NIH Publication No. 82–23, 1985) and the guidelines for the use of animals in experimental research as outlined by the Australian National Health and Medical Research Council. Traumatic brain injury was induced using the impact-acceleration model of diffuse traumatic brain injury (TBI) as previously described (Foda and Marmarou 1994). Animals in experimental research as outlined by the Australian National Health and Medical Research Council. Traumatic brain injury was induced using the impact-acceleration model of diffuse traumatic brain injury (TBI) as previously described (Foda and Marmarou 1994). Sham-operated controls (<em>n</em> = 9) were surgically prepared, but were not injured.

**Drug preparation and administration**

<em>N</em>-(nitro-2-phenoxypyphenyl)-methanesulphonamide (R805, marketed as Nimesulide, Cayman Chemical, Ann Arbor, USA) was suspended in DMSO and diluted into isotonic saline prior to administration. Animals were then administered nimesulide intraperitonally (6 mg/kg, <em>n</em> = 10) at 30 min after trauma and daily over a 10-day period. This dosage regimen is identical to that which has been used successfully in previous brain injury studies in rats (Wakita et al. 1999; Candelario-Jalil et al. 2000). The control group (<em>n</em> = 10) received an equal volume of vehicle using the same administration regimen.

**Assessment of cognitive performance**

The Barnes circular maze (Barnes 1979) as modified and described in detail by Fox et al. (1998) was used to assess spatial reference memory following diffuse traumatic brain injury. Animals were trained to locate a hidden escape tunnel in response to an aversive light and sound stimulus. The tunnel was placed directly beneath the platform after initiation of aversive stimuli was assessed daily over a 10-day post-traumatic period.

**Immunohistochemical techniques**

Following TBI, animals were anesthetized and killed by decapitation at 3 h, 6 h, 12 h, 24 h, 2 days, 3 days, 4 days, 5 days, 7 days, or 12 days (<em>n</em> = 6/ per time point) post-trauma. For immunoblotting assays, the brains were rapidly removed, and samples of parietal cortex and hippocampus were dissected. Extracts from brain samples were prepared by lysis in 100 mM HEPES buffer, pH 7.5, containing 10% sucrose, 1 mM EDTA, 1% Triton-X, 1 mM diithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 5 μg/ml aprotinin, and centrifuged at 10,000 g. Thirty micrograms of total protein of each sample was then loaded onto a 12% SDS-PAGE gel and after electrophoresis, protein was transferred to nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia). Blots were blocked with 5% nonfat milk in TBST (10 mM Tris, pH 7.2, 150 mM NaCl, 0.05% Tween 20) and incubated with antibody directed against COX-2 (polyclonal murine; 1:1000; Cayman Chemical Company, Ann Arbor, USA) for 1 h at room temperature. After washing the blot three times in PBS containing 0.1% Tween 20, the secondary antibody (goat anti-rabbit IgG-HRP; 1:10,000; Sigma, St. Louis, Mo.) was applied for a further 1 h at room temperature. The blots were then washed three times in PBS containing 0.1% Tween before being incubated in commercial enhanced chemiluminescence reagents (Amersham, Arlington Heights, Ill.) and exposed to Kodak BioMax ML film (Eastman Kodak Company, Rochester, N.Y.). To assess changes in estimated proteins quantitatively, the bands were analyzed by densitometry and quantified by computer analysis.

For immunohistochemistry, brains were removed at 3 days post-trauma (<em>n</em> = 3) and snap-frozen in liquid nitrogen and a cryostat used to obtain 8–10 μm coronal sections through the regions of interest. Slice-mounted slices were then incubated for 3 days at 4°C with polyclonal murine COX-2 antibody (1:2000; Cayman Chemical, Ann Arbor, USA) dissolved in PBS containing 3% of normal rat serum. After washing the slices in PBS, slices were incubated with IgG-HRP conjugated secondary antibody (1:400; Sigma-Aldrich) for 1 h at room temperature, and the subsequent immunocomplex visualized using diaminobenzidine as a chromogen in a peroxidase reaction (Sigma-Aldrich). To assist in the determination of the cellular localization of the label, the sections were counterstained with hematoxylin (Sigma-Aldrich). After rinsing, the slides were examined in a Leica DML LB microscope. Control conditions to assess nonspecific labeling included omission of the primary antibody, preabsorption of the COX-2 antibody or omission of hydrogen peroxide to block endogenous peroxidase activity. In all cases, the controls were negative.