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ACTIVATION OF CYCLO-OXYGENASE-2 CONTRIBUTES TO MOTOR AND COGNITIVE DYSFUNCTION FOLLOWING DIFFUSE TRAUMATIC BRAIN INJURY IN RATS

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SUMMARY

1. Post-traumatic inflammation may play a significant role in the development of delayed secondary brain damage following traumatic brain injury.

2. During post-traumatic inflammation, metabolic products of arachidonic acid, known as prostanoids (prostaglandins and thromboxanes) are released and aggravate the injury process. Prostanoid synthesis is regulated by the enzyme cyclo-oxygenase (COX), which is present in at least two isoforms, COX-1 (the constitutive form) and COX-2 (the inducible form).

3. In the present study, we examine the temporal and spatial profiles of COX-2 expression and the effects of the COX-2 inhibitor nimesulide on motor and cognitive outcome following diffuse traumatic brain injury in rats.

4. Adult male Sprague-Dawley rats were injured using the 2 m impact acceleration model of diffuse traumatic brain injury. At preselected time points after injury, animals were killed and the expression of COX-2 was measured in the cortex and hippocampus by western blotting techniques.

5. Increased expression of COX-2 was found in the cortex at 3 days and in the hippocampus as early as 3 h postinjury and this persisted for at least 12 days.

6. Administration of nimesulide (6 mg/kg, i.p.) at 30 min after injury and daily over a 10 day post-traumatic neurological assessment period resulted in a significant improvement compared with vehicle (2% dimethylsulphoxide diluted in isotonic saline)-treated controls in cognitive deficits, as assessed by the Barnes circular maze. There was also a significant improvement in motor dysfunction as assessed by the rotarod test on days 1 and 2 post-trauma compared with vehicle-treated controls.

7. These results implicate the involvement of COX-2 in cognitive and motor dysfunction following diffuse traumatic brain injury.

INTRODUCTION

It is now known that much of the brain damage produced by head impact is not the result of the initial trauma but, rather, develops over a period of hours to days after the primary event. Indeed, it is the complex secondary mechanisms initiated at the time of trauma that play an important role in the delayed progression of the brain damage. The identification of these secondary factors involved in the injury process is important because it may lead to new therapeutic strategies that improve functional outcome.[1] One of the secondary processes that may play a role in delayed neuronal death is post-traumatic inflammation, in which prostanoids, metabolic products of arachidonic acid, are among the pivotal regulators.[2] Prostanoid synthesis is regulated by cyclo-oxygenase (COX; or prostaglandin H synthase) that is present in at least two isoforms, COX-1, the constitutive form and COX-2, the inducible form.[3] Cyclo-oxygenase-2 regulates the key metabolic step in the biosynthesis of prostanoids, which are believed to play an important role in the control of cerebral circulation and neuronal signalling.[4] Moreover, because increased prostaglandin (PG) synthesis has been demonstrated in the brain following traumatic brain injury,[5] PG have been implicated in the pathogenesis of the physiological and morphological sequelae of traumatic brain injury (TBI). Consistent with this, it has recently been shown that an inhibition of prostanoid synthesis protects against neuronal damage following ischaemic brain injury.[6]

Increased COX-2 activity and enhanced release of prostanoids have been shown to be associated with the generation of highly reactive oxygen species (ROS).[4] Reactive oxygen species have potent deleterious effects on lipids, proteins and DNA and have been implicated in the development and progression of apoptotic cell death in the central nervous system.[7] Expression of COX-2 has also been shown to be an important determinant of cytotoxicity connected with inflammation.[3] Finally, induction of COX-2 has been demonstrated following experimental ischaemia,[8] after kainate treatment[9] and following lateral cortical impact injury.[10] However, no studies have examined the role of COX-2 in models of traumatic brain injury that have diffuse axonal injury as a major component. Accordingly, the aim of the present study was to examine the temporal and spatial

profiles of COX-2 expression and the effects of the COX-2 inhibitor nimesulide on motor and cognitive outcome following diffuse TBI in rats.

METHODS

Brain trauma model

All experimental protocols were approved by the James Cook University Experimental Ethics Committee and were conducted according to the Guidelines for the Use of Animals in Experimental Research as outlined by the National Health and Medical Research Council of Australia.

Traumatic brain injury was performed using the impact-acceleration model of diffuse TBI, as described previously.[11,12] Briefly, adult male Sprague-Dawley rats (350–450 g) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.), intubated and mechanically ventilated on room air using a Harvard Rodent Ventilator (Harvard Apparatus, Holliston, MA, USA). Rectal temperature was maintained at approximately 37°C with a thermostatically controlled heating pad. The skull was exposed by a midline incision and a stainless steel disc (10 mm in diameter and 3 mm in depth) was fixed rigidly with polyacrylamide adhesive to the animal's skull centrally between lambda and bregma. Rats were placed on a 10 cm deep foam bed and then subjected to the injury induced by dropping a 450 g brass weight a distance of 1 m onto the stainless steel disc. Such a model, as described previously in the original publications,[11,12] produces moderate diffuse axonal injury. Sham-injured animals underwent identical surgical procedures, but were not subject to impact injury.

Sample preparation and immunoblots

Following injury, animals were anaesthetized and killed by decapitation 3, 6, 12 and 24 h and 3, 4, 5, 7 and 12 days ($n = 5$ per time point) following diffuse TBI. The brains were removed rapidly and samples of parietal cortex and hippocampus were collected. Extracts from brain samples were prepared by lysis in 100 mmol/L HEPES buffer, pH 7.5, containing 10% sucrose, 1 mmol/L EDTA, 1% Triton-X, 1 mmol/L dithiothreitol (DTT), 1 mmol/L phenylmethylsulphonyl fluoride (PMSF), 1 μg/mL leupeptin, 1 μg/mL pepstatin A and 5 μg/mL aprotinin and centrifuged at 10 000 g. Thirty microgram total protein from each sample was then loaded onto a 12% sodium dodecyl sulphate–polyacrylamide gel and, after electrophoresis, protein was transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia, Castle Hill, NSW, Australia). Blots were blocked with 5% non-fat milk in TBST (10 mmol/L Tris, pH 7.2, 150 mmol/L NaCl, 0.05% Tween 20) and incubated in antibody directed against COX-2 (polyclonal murine IgG; 1 : 1000; Cayman Chemical, Ann Arbor, ME, USA) for 1 h at room temperature. After incubation, blots were washed three times in phosphate-buffered saline (PBS) containing 0.1% Tween 20, and the secondary antibody (goat antirabbit IgG-HRP; 1: 10 000; Sigma, St Louis, MO, USA) was applied for 1 h at room temperature. Blots were washed in PBS containing 0.1% Tween 20 three times and then incubated in commercial enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL, USA) and exposed to Kodak BioMax ML film (Eastman Kodak, Rochester, NY, USA). To quantitatively assess changes in estimated proteins, the appropriate bands were analysed by densitometry and quantified by computer analysis.

Drug preparation and administration

N-(Nitro-2-phenoxyphenyl)-methanesulphonamide (R805; marketed as Nimesulide; Cayman Chemical) was suspended in dimethylsulphoxide (DMSO) and further diluted into isotonic saline prior to administration (final concentration of DMSO 2%). Animals were then administered nimesulide intraperitoneally (6 mg/kg bodyweight; $n = 10$) or vehicle ($n = 10$) at 30 min after trauma and daily over a 10 day period.

Assessment of motor performance

Motor assessment was performed using the rotarod device, which has been described as being the most sensitive test to detect motor deficit in rodent brain injury.[13] Briefly, animals were placed on the rotarod device, consisting of a motorized rotating ensemble of 18 rods. The rotational speed of the device was increased from 0 to 30 r.p.m. at intervals of 3 r.p.m. every 10 s. The latency to fall from the rotating bars or to grip the rods and spin two consecutive rotations was recorded for each rat over a 10 day post-traumatic period.

Assessment of cognitive performance

The Barnes circular maze,[14] adapted from Bach *et al.*,[15] and described in detail by Fox *et al.*[16] was used to assess spatial reference memory following diffuse TBI. Animals were trained to locate a hidden escape tunnel, which was placed directly beneath one of 18 circular holes at the perimeter of a large circular platform. Latency, in seconds, to find the escape tunnel after initiation of aversive sound and light stimuli was tested daily over a 10 day post-traumatic period.
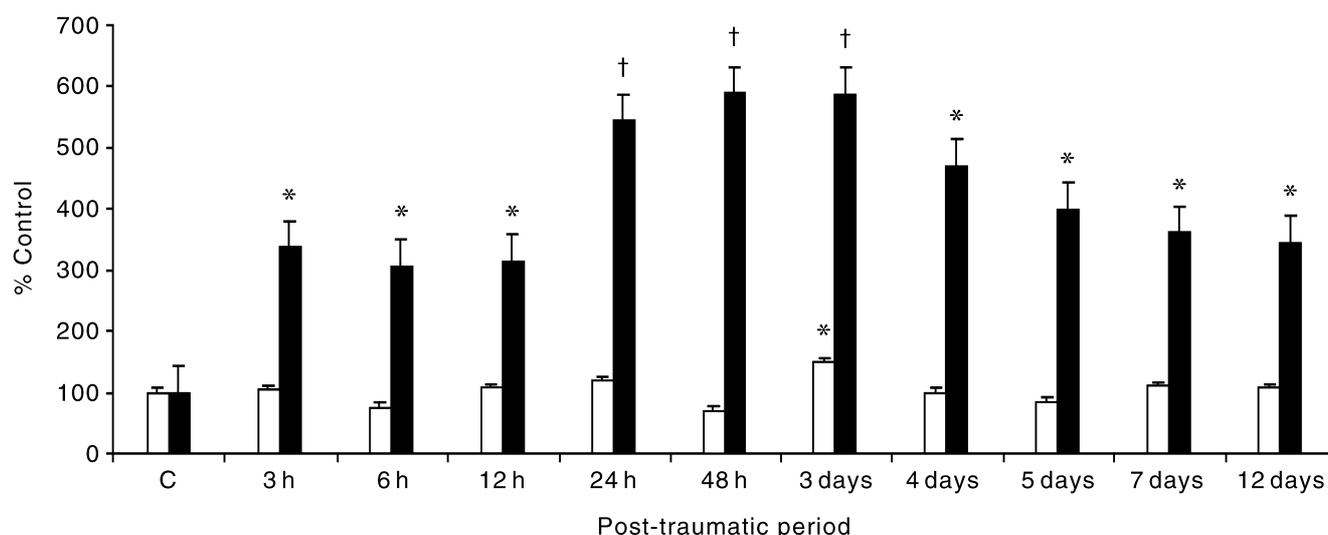

Fig. 1 Time-course of cyclo-oxygenase (COX)-2 levels in the cortex (□) and hippocampus (■) over a 12 day post-traumatic period following diffuse traumatic brain injury. Data are expressed as a percentage of control, where control is 100%. Bars represent the mean±SEM ($n = 5$ per time point). *$P < 0.01$, †$P < 0.001$ compared with control.

Fig. 2 Alterations in motor performance between animals treated with either nimesulide (cyclo-oxygenase-2 inhibitor; ●) or vehicle (2% dimethyl-sulphoxide diluted in isotonic saline; ■) over a 10 day assessment period following diffuse traumatic brain injury in rats. Data are the means±SEM ($n = 10$ per time point). *$P < 0.05$ between treatment groups.
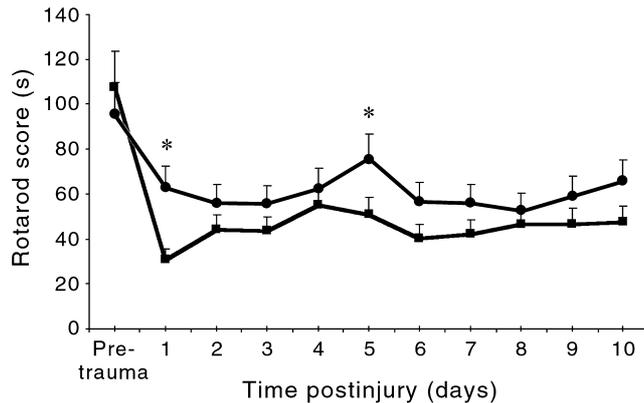

Fig. 3 Alterations in motor performance between animals treated with either nimesulide (cyclo-oxygenase-2 inhibitor; ●) or vehicle (2% dimethylsulphoxide diluted in isotonic saline; ■) over a 10 day assessment period following diffuse traumatic brain injury in rats. Data are the means±SEM ($n = 10$ per time point). *$P < 0.05$ between treatment groups.

Statistical analysis

Statistical significance for western blots was determined by analysis of variance (ANOVA) followed by Student–Newman–Keul's post hoc tests. For analysis of behavioural data, a repeated-measures ANOVA was used to determine group main effects. The least significant pairwise multiple comparison post hoc test was used to determine group differences on specific days after injury. The level of significance was set at $P < 0.05$.

RESULTS

Cyclo-oxygenase-2 protein levels in brain structures following TBI

Figure 1 shows the time-course of COX-2 expression in the cortex and hippocampus following acceleration-induced impact injury. The immunoreactivity for COX-2 is increased by approximately 50% in the cortex at 3 days postinjury ($148 \pm 16\%$) compared with control animals. An increase in hippocampal COX-2 immunoreactivity occurred within 3 h, with a maximal level at 48 h ($588 \pm 68\%$ *vs* control values) and persisted until the end of the 12 day observation period (Fig. 1).

Motor function following TBI

Traumatic brain injury caused a significant motor deficit in vehicle-treated control animals, with the nadir noted at 24 h postinjury (30 ± 8 s; $P < 0.001$ *vs* pre-injury values of 98 ± 16 s). The motor deficit persisted for the full 10 day post-traumatic assessment period (Fig. 2). Nimesulide treatment reduced the injury induced motor deficit such that a statistically significant improvement was observed at 24 h and 5 days post-trauma (Fig. 2).

Cognitive function following TBI

Prior to injury, mean latency time for animals to locate the Barnes maze tunnel was 9 ± 1 s. Following injury, the latency to locate the tunnel increased significantly ($P < 0.001$) and peaked at 24 h and 7 days post-trauma (69 ± 18 and 37 ± 9 s, respectively). Treatment with nimesulide significantly improved cognitive performance at almost all time points post-trauma when compared with vehicle-treated controls (Fig. 3).
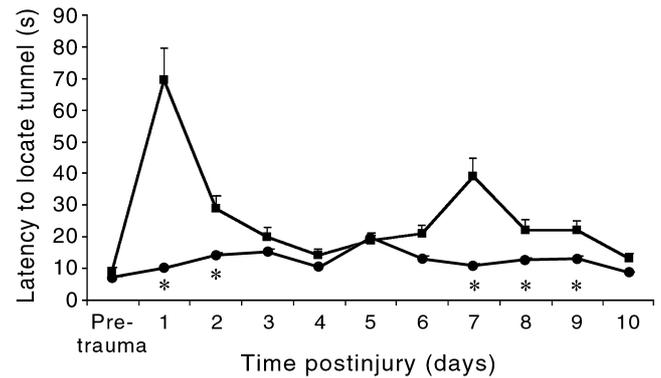
DISCUSSION

In the present study, we have demonstrated that diffuse TBI in rats increases the expression of COX-2 protein in the cortex and hippocampus. Such an increase in COX-2 protein expression in the hippocampus started as early as 3 h postinjury and persisted for 12 days. Administration of a specific inhibitor of COX-2, namely nimesulide, during this time period significantly reduced cognitive deficit and improved motor performance.

Inhibition of PG synthesis following brain injury has been reported as neuroprotective in experimental studies of focal ischaemia in rats,[6] concussive brain injury in cats,[17] fluid-percussion-induced neurotrauma in rats[18] and in clinical neuro-surgery.[19] Increases in PG are a consequence of COX-2 activity and may have direct effects on further secondary injury mechanisms, such as free radical generation[20] and neurotransmitter release.[21] Following lateral cortical impact injury, increased COX-2 levels reach a plateau in both the ipsilateral cortex and hippocampus at 24 h–3 days.[10,22] Our results demonstrate a similar trend of COX-2 protein expression in hippocampus, but of longer duration (up to 12 days) and of higher amplitude. Furthermore, significantly increased COX-2 levels in the cortex were shown in the present study only on day 3 postinjury. Differences between the lateral cortical impact injury model used in previous studies and the acceleration (weight drop) impact injury model used in the present study may account for such variance. We assume that the extended time-course for COX-2 immunoreactivity in the hippocampus observed in the present study could be a consequence of either a relatively long half-life of the protein or its continued transcription. Moreover, the differences in COX-2 expression between the cortex and hippocampus may be accounted for by regionally specific responses to trauma.

The specific COX-2 inhibitor nimesulide was shown to reduce white matter damage in chronic cerebral ischaemia[23] and limit kainate-induced oxidative damage in the rat hippocampus.[24] In the present study, animals receiving nimesulide demonstrated significant attenuation of cognitive deficits compared with non-injured animals, but the beneficial effects on motor performance were much less profound. In contrast with our findings, Dash *et al.*[10] demonstrated that celecoxib, another specific COX-2 inhibitor, worsened

motor performance without affecting cognitive outcome following lateral cortical impact injury in rats. Such an inconsistency between results may be a consequence of the different pharmacological profiles of the two COX-2 inhibitors or a function of the different injury models that were used in the two studies. Taken together, our findings support the involvement of COX-2 in cognitive and motor dysfunction following diffuse TBI.

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