Window of Opportunity for Neuroprotection with an Antioxidant, Allene Oxide Synthase, after Hypoxia–Ischemia in Adult Male Rats

Sam Mathai,^{1,2} Alistair J. Gunn,² Ralph Andrew Backhaus^{3,4} & Jian Guan¹

¹ The Liggins Institute, The University of Auckland, Auckland, New Zealand

² Department of Physiology, Faculty of Medical and Health Sciences, The University of Auckland, Auckland, New Zealand

³ Pharma Pacific, Phoenix, AZ, USA

⁴ Pacific Brands, Herald Island, Auckland, New Zealand

Keywords

Allene Oxide Synthase; Neuroprotection; Rats and hypoxic–ischemic brain injury.

Correspondence

Jian Guan, The Liggins Institute, The University of Auckland, Private Bag 92019, Auckland, New Zealand.

Tel.: +64-9-923-6134;

Fax: +64-9-373-7497;

E-mail: j.guan@auckland.ac.nz

Received 30 April 2012; revision 18 June

2012; accepted 14 August 2012.

doi: 10.1111/cns.12004

SUMMARY

Aims: Oxidative stress is an early event in the cascade leading in neuronal damage after hypoxic–ischemic (HI) brain injury. In the present study, we examined the dose response and window of opportunity for neuroprotection after HI injury with Allene Oxide Synthase (AOS), an anti-oxidative enzyme of the member of cytochrome P450 family. **Methods:** Adult male rats received intra-cerebro-ventricular infusions of either saline (vehicle) or AOS (1 μ g or 10 μ g or 100 μ g per rat, intracerebroventricular $n = 16$ all groups) either 45 min or 3 h after unilateral HI brain injury. Brains were collected 5 days later. The extent of brain damage, neuronal survival, apoptosis, and glial reactions were assessed in the striatum, hippocampus, and cortex. **Results:** Allene Oxide Synthase was associated with reduced neuronal damage scores when given 45 min, but not 3 h, after HI injury ($P < 0.0001$) in all brain regions. AOS treatment (10 μ g) improved neuronal survival in the striatum, cortex, and hippocampus ($P < 0.05$, $P < 0.001$) and reduced the microglia reaction ($P < 0.05$) and numbers of caspase-3-positive cells in the hippocampus ($P < 0.01$). **Conclusions:** Early blockade of oxidative stress after HI injury reduces inflammatory response, neuronal necrosis, and apoptosis. The neuroprotective effects of AOS were time of administration-dependent suggesting a relatively restricted window of opportunity for acute brain injury.

Introduction

Hypoxic–ischemic (HI) brain injury, for example, after cardiac arrest, remains a major cause of death and long-term disability [1]. Considerable evidence suggests that the sudden increase in tissue oxygenation immediately after ischemic injury leads to excess free radical production, lipid peroxidation, mitochondrial damage, and inflammatory reaction [2–4], ultimately promoting both necrotic and apoptotic neuronal death [5,6]. Anti-oxidative interventions can reduce oxidative stress and improve recovery from HI injury in experimental paradigms, but none have yet been successfully translated into clinical practice [7,8]. Although this lack of success is undoubtedly multifactorial, oxidative damage appears to occur primarily during reperfusion/re-oxygenation, and thus, a short window of opportunity for intervention may be an important factor contributing to the prevention of HI brain injury.

Allene oxide synthase (AOS), an atypical member of P450 cytochrome (CYP) superfamily [9], is a plant enzyme that metabolizes fatty acid hydroperoxides to make intermediates used for the production of small quantities of jasmonates that act as signaling compounds for plant defense [10]. Physiologically, AOS is usually

localized in plant chloroplasts and is produced in minute quantities [11]. A novel form of AOS, discovered in the guayule rubber plant, is localized in rubber particles and is produced in enormous quantities [12]. This unusual rubber particle isoform is notable for its high catalytic activity, its ability to metabolize a wide range of fatty acid hydroperoxides, including arachidonic hydroperoxide [12–14], and has the ability to act as a potent antioxidant in diverse biological systems [15]. Moreover, its antioxidant properties were robust enough to prevent heart damage following ischemia–reperfusion injury in a perfusion chamber system [16]. Given that HI injury induced oxidative stress is also a key mechanism associated with neuronal death, we tested the protective effects and the window of opportunity of this AOS isoform after HI injury in adult rats.

Methods

Experimental Procedures

All experimental procedures were approved by the animal ethics committee of the University of Auckland. Every effort was made to minimize animal suffering and to reduce the number of animals

used for the study. Adult male Wistar rats (280–310 g, 60–70 days of age) were obtained from the animal resources unit of the University of Auckland. A guide cannula to deliver AOS to the right lateral ventricle was placed 3 days before induction of HI injury. Briefly, rats were anesthetized using isoflurane (3% isoflurane/oxygen). The cannula (21 G $\times$ 6 mm) was stereotactically placed on the top of the dura on the right through a hole drilled at the coordinates of 1.5 mm from the midline and +7.5 mm anterior–posterior (A–P) from the interaural zero plane and fixed with dental cement [17].

Three days after cannulation, the rats were re-anesthetized with 3% isoflurane/oxygen, and the right common carotid artery was exposed and ligated twice with 0/3 silk sutures. Two hours after the ligation, rats were placed in an incubator with controlled relative humidity ($90 \pm 5\%$) and temperature ($31 \pm 0.5^\circ\text{C}$). The oxygen concentration in the incubator was rapidly reduced to 6% by introducing pure nitrogen and then maintained at

$6 \pm 0.2\%$ by a mixture of pure nitrogen and air for a further 15 min. The oxygen concentration in the incubator was constantly monitored.

Drug Preparation and Administration

Either 45 min or 3 h after HI injury, the rats were re-anesthetized with 3% of isoflurane/oxygen, and a 27-G infusion needle, connected to a 100- μL Hamilton syringe by a catheter (internal diameter: 0.35 mm), was inserted through the pre-fixed cannula. AOS (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in normal saline at three concentrations (1 μg or 10 μg or 100 $\mu\text{g}/20 \mu\text{L}$). Either AOS or an equal volume of saline (20 μL) was infused into the right lateral ventricle at 1 $\mu\text{L}/\text{min}$ controlled by a microdialysis infusion pump (CMA/100; Carnegie Medicin, Stockholm, Sweden). The rats were allowed to recover in the holding room for 5 days and then killed for tissue collection.

Figure 1 The photographs show thionin/acid fuchsin stained brain sections from rats treated with vehicle (left panel) or with 10 μg Allene Oxide Synthase (AOS) (right panel) 45 min after hypoxic–ischemic injury in the striatum (**A, B**), CA1-2 (**C, D**), CA3 (**E, F**), the CA4, and dentate gyrus of hippocampus (**G, H**) and the cortex (**I, J**). Dead and damaged neurons were markedly fewer in the AOS-treated group. Arrows indicate neurons showing ischemic cell change (pink acidophilic cytoplasm and contracted nuclei), and arrowheads indicate pan-necrotic areas in the brain. Scale bar = 40 μm (**A–J**).

Figure 2 The effect of Allene Oxide Synthase (AOS) treatment on tissue damage scores when given 45 min (A) or 3 h (B) after the hypoxic-ischemic insult. Compared to the vehicle-treated group, neuronal damage scores in the AOS-treated groups were significantly less in all brain regions examined. When the treatment was delayed by 3 h, there was no reduction in neuronal damage scores. Data are mean $\pm$ SEM, * P < 0.05, STR, striatum; DG, dentate gyrus; CTX, cortex.

Tissue Preparation, Staining, and Assessment

Tissue collection and staining have been described previously [17,18]. Briefly, rats were deeply anesthetized using pentobarbital (125 mg/kg, intraperitoneally), and the brains were transcardially perfused with normal saline, followed by 10% neutral buffered formalin. The brains were removed and further fixed in the formalin for another 2 days, then processed and paraffin-embedded. Coronal sections (6 μ m) were cut from the striatum, cerebral cortex, and hippocampus [19] and mounted on albumen-chrome alum precoated glass slides. Adjacent sections were collected from 6-mm A-P for analysis of the striatum and the cortex and from both 4.5 and 4.2 mm A-P for analysis of the hippocampus and two further cortical levels [20] and stained with thionin/acid fuchsin.

Brain tissues showing selective neuronal death, cellular reaction, and/or pan necrosis were considered as damaged [19]. Selective neuronal death was identified by the presences of acidophilic (pink) cytoplasm and contracted nuclei [17,21,22]. The severity of brain damage was assessed at three levels in the lateral cortex; at two levels in the hippocampus and the dentate gyrus (DG); and at one level in the striatum using a 4-point scoring system (0 = no damage; 1 = few cells damaged; 2 = <half of tissue damaged; 3 = >half of tissue damaged; and 4 = almost no tissue survived). The average tissue damage scores in different brain regions were used for data analysis [19,23]. All histological assessments were carried out by an experimenter blinded to the treatments.

Immunohistochemical Staining and Assessments

The extent of neuronal survival, apoptotic cell death, and inflammatory response were evaluated in the groups treated with vehicle or 10 μ g AOS using immunohistochemical staining. The following primary antibodies were used: monoclonal mouse anti-NeuN for labeling surviving neurons (1:200; Chemicon International Inc., Temecula, CA, USA); rabbit polyclonal anti-neuronal NOS (1:200; Abcam, Sapphire Bioscience (NZ) Ltd., Hamilton, New Zealand) for nNOS-containing GABA interneurons; rabbit polyclonal cleaved anti-Caspase-3 (ASP-175) for apoptotic cells (1:200; Cell Signalling Technology, Denver, MA, USA); and goat polyclonal anti-Iba-1 for reactive microglia (1:200; Abcam). All slides were de-paraffinized in xylene, rehydrated using a graded ethanol series, and washed with 0.1 M phosphate buffered saline except in the case of Iba-1 where Tris-buffered saline was used. The sections were then treated with 1% H_2O_2 in methanol for 20 min in darkness to quench endogenous peroxidase. Antigen retrieval was performed by keeping the slides in 10 mM sodium citrate buffer (pH 6.0) at near boiling temperature (95°C) for 20 min. The slides were washed and incubated with appropriate primary antibodies at 4°C for 48 h and secondary antibodies for overnight. PBS-washed slides were then incubated with ExtrAvidin® (Sigma-Aldrich Pty, Ltd., Sydney, Australia) for 3 h at room temperature. Finally, sections were treated with 3, 3', diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Pty, Ltd.) + 0.01% H_2O_2 to get the brown reaction product. Alcohol-dehydrated sections were then cleared in xylene and cover-slipped with DPX.

The numbers of NeuN, nNOS, caspase-3-, and Iba-1-positive cells were counted on the ipsilateral (damaged) and contralateral (undamaged) hemispheres using Stereo Investigator software (MBF Bio Science, Williston, VT, USA) by light microscopy (Nikon 80i, Tokyo, Japan). All immunohistochemical counting was completed by an assessor blinded to this study. Neuronal survival was assessed by counting NeuN-positive cells in the lateral cortex, CA1-2, CA3, CA4, and DG subregions of the hippocampus and the striatum. The number of nNOS-positive cells was counted in the striatum, hippocampus as one region, and lateral cortex. Iba-1-positive microglia and caspase-3-positive cells were counted in the hippocampus representatively. First, a contour was drawn at 2 $\times$ magnification surrounding the area of interest. The cells were counted after random sampling with stereo investigator at 40 $\times$ magnification and applying a fractionator probe consisting of a counting frame for object inclusion/exclusion. Area was measured, and numbers of cells were automatically recorded by the software. Number of immunopositive cells were converted to density (cells/mm²) by applying the equation [(Total markers counted/Number of sampling sites $\times$ counting frame area)] $\times$ 10⁶ [24]. The Right/Left ratio of immunopositive cell counts was calculated for each animal.

Statistics

The effect of AOS on tissue damage scores and neuronal survival (NeuN and nNOS) was analyzed by two-way ANOVA followed by Bonferroni *post hoc* tests, with brain regions treated as a dependant factor (GraphPad 5 PRISM®, GraphPad Software, Inc., La Jolla,

CA, USA). Caspase-3-positive cells and Iba-1-positive microglia in the vehicle and 10- μ g groups were compared using *t*-tests (Graph-Pad 5 PRISM®). Data are presented as mean $\pm$ SEM. Statistical significance was accepted at $P < 0.05$.

Results

Effect of Allene Oxide Synthase on Tissue Damage Scores

Hypoxic-ischemic injury resulted in severe tissue damage in the ipsilateral (ligated) side hemispheres 5 days after the insult, with no histological change in the contralateral side of hemisphere. Morphologically, the group treated with the vehicle showed widespread neuronal death where the dead neurons were identifiable with pink acidophilic cytoplasm and contracted nuclei, and/or pan necrosis with marked cellular reaction (examples are shown in Figure 1A,C,E,G,I). In

comparison, the morphological changes were milder in the group treated with AOS in all the brain regions assessed (Figure 1B,D,F,H,J).

Tissue damage scores were significantly less in the group treated with AOS 45 min after HI injury ($P < 0.0001$, $F = 19.21$ [3,341], $n = 15$ –16) compared to the vehicle-treated group. There was no difference between brain regions within the groups, and the treatment effect was not dependent on the brain regions. Bonferroni post-tests suggested that the treatment with 10 μ g AOS significantly prevented neuronal damage in the lateral cortex, the CA1-2, CA3, CA4, and DG subregions of the hippocampus ($n = 15$, $P < 0.05$) (Figure 2A). The neuroprotective effects of 100 μ g AOS were only associated with the striatum, the CA4, and DG of hippocampus and lateral cortex ($n = 15$, $P < 0.05$) compared to the vehicle-treated groups ($n = 16$). There was no statistical difference in tissue damage scores between the groups treated with 1 μ g AOS and the vehicle.

Figure 3 The photographs show NeuN immunopositive neurons after either vehicle (left panel) or 10 μ g Allene Oxide Synthase (AOS) treatment (right panel) in the striatum, hippocampus, and cortex. There were fewer NeuN-positive cells in the vehicle-treated group and many of the neurons appeared pycnotic. The enlarged images (the insert) show pycnotic neurons in the cortex (I). The NeuN-positive neurons after the AOS treatment were more and morphologically normal (right panel). The insert (J) shows the morphology of NeuN-positive neurons after AOS treatment. Scale bar = 40 μ m (A–J).

Figure 4 The effect of Allene Oxide Synthase (AOS) given 45 min after hypoxic–ischemic injury on neuronal survival. The R/L ratio of the NeuN-positive cells after 10 µg AOS treatment was significantly increased compared to vehicle treatment in all brain regions examined. The data are presented as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, STR, striatum; DG, dentate gyrus; CX, cortex.

In contrast, there was no significant difference in tissue damage scores between the treatment groups when AOS treatment was delayed until 3 h after HI injury (Figure 2B, $n = 16$).

Effects of Allene Oxide Synthase on Neuronal Survival

Hypoxic–ischemic injury induced a unilateral loss of NeuN-positive neurons in the ipsilateral striatum, the subregions of hippocampus, and lateral cortex of the vehicle-treated group. Morphologically, the NeuN-positive neurons were contracted with densely stained nuclei, and typically, extensive areas of necrotic tissue damage were seen surrounding surviving neurons (Figure 3A,C,E,G,I). The effect of AOS on neuronal survival was only examined in the group receiving 10 µg AOS 45 min after HI injury. Compared to vehicle treatment, NeuN-positive neurons in the AOS-treated groups showed normal morphology (Figure 3B,D,F,H,J).

Treatment with AOS was associated with a significantly increased R/L ratio of neuronal counts ($P < 0.0001$, $F = 71$ [1, 69] $n = 5$ –10, Figure 4). There was no difference between the brain regions, and the treatment effect was independent of brain regions. Bonferroni post-test showed significantly improved neuronal survival in the striatum ($P < 0.05$), CA1-2 ($P < 0.001$), CA3 ($P < 0.001$), CA4 ($P < 0.01$), DG ($P < 0.05$), and lateral cortex ($P < 0.001$, Figure 4).

Effects of Allene Oxide Synthase on nNOS-Positive Neurons

Neuronal NOS-positive neurons, the bipolar interneurons, were evenly distributed throughout the cortex, striatum, and the hippocampus. Morphologically, nNOS-positive neurons were smaller, dense, and had few dendrites in the rats treated with the vehicle (Figure 5A). HI injury was associated with severe loss of nNOS-positive neurons. Compared to the vehicle-treated group, R/L ratio of nNOS neurons in 10 µg AOS-treated group was significantly higher (Figure 6A, $P < 0.001$, $F = 18.9$ [1, 64], $n = 9$ –

14), particularly in the hippocampus ($P < 0.05$) with improved morphology (Figure 5A,B).

Effects of Allene Oxide Synthase on Caspase-3-Mediated Neuronal Death

Hypoxic–ischemic injury was associated with the induction of caspase-3-positive neurons throughout the ipsilateral striatum, all subregions of the hippocampus, and cortex. Figure 5C shows the abundant distribution of caspase-3-positive neurons in the CA3 subregion of the hippocampus after vehicle treatment. In contrast, there were almost no caspase-3-positive neurons in the CA3 subregions of the hippocampus after AOS treatment (Figure 5D). Compared to the vehicle-treated group, the R/L ratio of caspase-3-positive neurons in the hippocampus was significantly lower in the 10 µg AOS-treated group (Figure 6B, $P < 0.01$).

Effects of Allene Oxide Synthase on Microglial Response

There was marked induction of darkly stained Iba-1-positive microglia throughout ipsilateral hemispheres after the injury, especially in the hippocampus (Figure 5E,F), which could be identified by their characteristic amoeboid morphology. The R/L ratio of microglia in the hippocampus after AOS treatment was significantly reduced compared to vehicle (Figure 6C, $P < 0.05$).

Discussion

The current study demonstrates for the first time that central administration of a potent antioxidant, AOS, 45 min after HI injury, reduced brain injury in a dose-dependent manner. Treatment with AOS 45 min after HI injury improved neuronal survival and attenuated caspase-3-mediated apoptosis and microglial reactivity, suggesting that protection with AOS may be mediated by inhibiting inflammation and apoptotic cell death. The window of opportunity for AOS to prevent neuronal death was relatively limited, with no statistically significant protection when AOS was given 3 h after HI.

The model of HI injury used in the present study induces unilateral ischemic–reperfusion brain damage. Carotid artery ligation induces only a relative ischemia in the area supplied by the middle cerebral artery, owing to cerebrovascular autoregulation that compensates for loss of the blood supply. The brief hypoxia which damages cerebral regulation is essential to trigger HI injury within the territory of ipsilateral middle cerebral artery. The sudden restoration of oxygen supply at the end of the hypoxic insult leads to the accumulation of free radicals. The oxidative stress subsequent to re-oxygenation is a major contributor to mitochondrial damage after HI injury, which in turn triggers the early stages of the cascade of caspase-mediated apoptotic and necrotic neuronal damage [25–28].

A single dose of AOS given 45 min after HI injury significantly reduced tissue damage, indicating a reduction of necrotic neuronal death and its consequences, tissue pan necrosis, and glial reaction. The treatment effect was found in all brain regions examined, including the lateral cortex, the subregions of hippocampus, and the striatum with the optimal dose (10 µg per rat). The treatment effects of AOS were clearly dose-dependent as 1 µg AOS was not protective and 100 µg AOS was effective only in some brain

Figure 5 The photographs show nNOS-positive neurons in the striatum (**A, B**), caspase-3-positive apoptotic neurons (**C, D**), and Iba-1-positive microglia (**E, F**) in the CA3 subregions of the hippocampus after vehicle (left panel) or 10 μ g Allene Oxide Synthase (AOS) treatment (right panel). Hypoxic–ischemic (HI) injury resulted in severe loss of nNOS-positive neurons in the striatum (**A**), hippocampus, and cortex, and most of the survived neurons were pycnotic. AOS treatment was associated with greater survival of nNOS-positive neurons, with improved morphology (**B**). HI injury was associated with increased caspase-3-positive apoptotic cells (**C**) and inflammatory reaction with massive infiltration of microglia in the hippocampus (**E**). AOS treatment was associated with a marked reduction of caspase-3 expression (**D**) and reduced inflammatory responses (**F**). Scale bar = 40 μ m.

regions. However, AOS was not protective when treatment was delayed by 3 h. The data strongly suggest that the time of administration of AOS is critical for preventing neurons from ischemic–reperfusion damage. This is consistent with the evidence that oxidative stress is an early event that is strongly linked with initial and rapid re-oxygenation after ischemia–reperfusion [17,29].

The treatment effects also appeared to vary between brain regions. It is known that the neurons in the subregions of the hippocampus are more sensitive to the ischemic injury. This may in part contribute to the ineffectiveness of high-dose AOS in the hippocampus. This finding is biologically highly plausible and suggests that early treatment with antioxidative treatment is essential for blocking free-radical injury. Realistically, only a few percentage of patients are able to receive hospital treatment within 45 min after acute brain injury.

Hypoxic–ischemic brain injury is associated with both necrotic neuronal loss due to osmotic swelling/rupture of cell membranes and progressive apoptotic death, as shown by cytoplasmic and nuclear condensation and activation of caspases [23]. In the pres-

ent study, the HI injury induced a significant loss of neurons, including nNOS-containing GABA interneurons and the activation of microglia and elevation of caspase-3-mediated apoptosis. We have previously reported continuing apoptosis over prolonged periods in the same rat model as used here [30]. This is also consistent with data from others that cell loss can continue for months after ischemic injury [31], particularly after moderate insults [32]. AOS (10 μ g) treatment significantly improved neuronal survival and dramatically inhibited microgliosis and neuronal apoptosis in the hippocampus to the level similar to those in the undamaged (contra-lateral) side of hippocampus.

Microgliosis has been used as a measure for inflammation after HI brain injury and is highly associated with the development of necrotic neuronal death and tissue infarction in adult rats [17]. In experimental models, inflammation is associated with the release of many toxic substances, including glutamate, hydrogen peroxide, and tumor necrosis factor α (TNF α) [33–35]. A limitation of the present study is that we did not evaluate specific free radicals and pro-inflammatory factors that are involved in HI-induced mi-

Figure 6 R/L ratio of nNOS-positive neurons (A) in the striatum, hippocampus, and cortex; caspase-3-positive neurons (B); and microglial cells (C) in the hippocampus. Compared to vehicle, 10 µg Allene Oxide Synthase treatment was associated with a marked increase in the R/L ratios of nNOS neurons, particularly in the hippocampus, and significant reductions in both caspase-3-positive neurons and microglial cells in the hippocampus. Data are mean $\pm$ SEM, * P < 0.05, ** P < 0.001.

croglial reaction. Nevertheless, the robust suppression of microglial induction associated with AOS suggests that at least in part, neuroprotection with AOS is likely to be mediated by inhibiting the microglial reaction.

Similarly, the effect of AOS treatment on caspase-3-mediated apoptosis was relatively greater than the effect on neuronal survival and overall tissue damage. While early AOS treatment reduced tissue damage by approximately half, neuronal apoptosis was almost completely blocked after AOS treatment. One well-known difference between apoptotic and necrotic pathways is that neuronal death through apoptosis often develops later than that through necrosis [36]. This may contribute to a broader window of opportunity for preventing apoptotic neuronal death when AOS was given 45 min after HI injury.

We have previously reported the loss of nNOS-containing GABA interneurons after HI injury [19]. Treatment with AOS improved the survival of these nNOS-positive neurons and maintained their morphological integrity. Nitric oxide (NO) is an important inhibitory neurotransmitter and has been reported to play a major role in the neuronal rescue in the present paradigm of HI injury [37], likely by promoting vasodilation [38] and improving cerebral blood flow during hypoxia [39]. Conversely, inhibition of NO synthesis increases focal ischemic infarction in rats [40] and neuronal injury in rats by impairing autoregulation and reduction of local cerebral blood flow [41]. Thus, the protective effects of AOS on nNOS-containing neurons may have also secondarily contributed to overall neuroprotection in our study.

While the precise mechanisms of AOS in protecting neuronal cells from HI injury are not known, it most likely involves the rapid disposal of lipid hydroperoxides generated during reperfusion/re-oxygenation. In the guayule plant, where rubber particles are very susceptible to oxidative attack, AOS evolved as a means to eliminate lipid hydroperoxides to suppress their toxicity [15]. In mammals, removal of lipid hydroperoxides relies on glutathione peroxidase 4 (GPx4), which is essential for survival. Knockout of GPx4 in mice leads to rapid embryo death [42,43], and inactivation of an inducible GPx4 results in the accumulation of cellular lipid hydroperoxides that trigger apoptosis-inducing factor (AIF)-mediated cell death [44]. Moreover, overexpression of GPx4 in neuronal cells increases the survival of neurons exposed to oxidative stress and reduces neuronal apoptosis [45]. Taken together, the free radical onslaught of lipid hydroperoxides generated by HI injury may overwhelm endogenous antioxidant systems and GPx4 levels in the mammalian brain rendering them inadequate to prevent neuronal death. These results suggest that the plant robust AOS isoform used in these experiments offers potent antioxidant augmentation that can prevent neurons from necrotic and apoptotic injury by suppressing inflammation, if it is administered in a timely fashion.

Acknowledgments

The project was financially supported by Pacific Brand Ltd. The authors thank Rani Wilson for excellent technical support.

Conflict of Interest

The data presented in this manuscript are not published or submitted elsewhere for publication. As an employee of Pacific Brand, Auckland, New Zealand, Dr A Backhaus shares financial interests with the company.

References

1. Arciniegas DB. Hypoxic-ischemic brain injury: addressing the disconnect between pathophysiology and public policy. *NeuroRehabilitation* 2010;**26**:1–4.
2. Niizuma K, Endo H, Chan PH. Oxidative stress and mitochondrial dysfunction as determinants of ischemic neuronal death and survival. *J Neurochem* 2009;**109**(Suppl 1):133–138.
3. Inder TE, Volpe JJ. Mechanisms of perinatal brain injury. *Semin Neonatol* 2000;**5**:3–16.
4. Bagenholm R, Nilsson UA, Kjellmer I. Formation of free radicals in hypoxic ischemic brain damage in the neonatal rat, assessed by an endogenous spin trap and lipid peroxidation. *Brain Res* 1997;**773**:132–138.
5. Northington FJ, Ferriero DM, Graham EM, Traystman RJ, Martin LJ. Early neurodegeneration after hypoxia-ischemia in neonatal rat is necrosis while delayed neuronal death is apoptosis. *Neurobiol Dis* 2001;**8**:207–219.
6. Guan J, Bennet L, Gluckman PD, Gunn AJ. Insulin-like growth factor-1 and post-ischemic brain injury. *Prog Neurobiol* 2003;**70**:443–462.
7. Silbergleit R, Haywood Y, Fiskum G, Rosenthal RE. Lack of a neuroprotective effect from N-acetylcysteine after cardiac arrest and resuscitation in a canine model. *Resuscitation* 1999;**40**:181–186.
8. Kaur C, Ling EA. Antioxidants and neuroprotection in the adult and developing central nervous system. *Curr Med Chem* 2008;**15**:3068–3080.
9. Song WC, Brash AR. Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. *Science* 1991;**253**:781–784.
10. Feussner I, Wasternack C. The lipoxygenase pathway. *Ann Rev Plant Biol* 2002;**53**:22.
11. Song WC, Funk CD, Brash AR. Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. *Proc Natl Acad Sci USA* 1993;**90**:8519–8523.
12. Pan Z, Durst F, Werck-Reichhart D, et al. The major protein of guayule rubber particles is a cytochrome P450. Characterization based on cDNA cloning and spectroscopic analysis of the solubilized enzyme and its reaction products. *J Biol Chem* 1995;**270**:8487–8494.
13. Pan Z, Camara B, Gardner HW, Backhaus RA. Aspirin inhibition and acetylation of the plant cytochrome P450, allene oxide synthase, resembles that of animal prostaglandin endoperoxide H synthase. *J Biol Chem* 1998;**273**:18139–18145.
14. Li L, Chang Z, Pan Z, Fu ZQ, Wang X. Modes of heme binding and substrate access for cytochrome P450 CYP74A revealed by crystal structures of allene oxide synthase. *Proc Natl Acad Sci USA* 2008;**105**:13883–13888.
15. Backhaus RA, Pan Z, Herickhoff LA. Enzymatic antioxidant of allene oxide for lipid peroxidation in biological systems. US Patent: 6,132,711, 2000.
16. Backhaus RA. Cardioprotective therapies based on enzymatic elimination of lipid peroxides by allene oxide synthase. US Patent: 7,157,082 2007.
17. Guan J, Williams C, Gunning M, Mallard C, Gluckman P. The effects of IGF-1 treatment after hypoxic-ischemic brain injury in adult rats. *J Cereb Blood Flow Metab* 1993;**13**:609–616.
18. Guan J, Williams CE, Skinner SJ, Mallard EC, Gluckman PD. The effects of insulin-like growth factor (IGF)-1, IGF-2, and des-IGF-1 on neuronal loss after hypoxic-ischemic brain injury in adult rats: evidence for a role for IGF binding proteins. *Endocrinology* 1996;**137**:893–898.
19. Guan J, Gunn AJ, Sirimanne ES, et al. The window of opportunity for neuronal rescue with insulin-like growth factor-1 after hypoxia-ischemia in rats is critically modulated by cerebral temperature during recovery. *J Cereb Blood Flow Metab* 2000;**20**:513–519.
20. Paxinos G, Watson CD. *The rat brain stereotaxic coordinations*. New York: Academic Press, 1982.
21. Brown AW, Brierley JB. Anoxic-ischaemic cell change in rat brain light microscopic and fine-structural observations. *J Neurol Sci* 1972;**16**:59–84.
22. Auer RN, Kalimo H, Olsson Y, Siesjö BK. The temporal evolution of hypoglycemic brain damage. II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. *Acta Neuropathol* 1985;**67**:25–36.
23. Guan J, Thomas GB, Lin H, et al. Neuroprotective effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine-proline-glutamate (GPE) following intravenous infusion in hypoxic-ischemic adult rats. *Neuropharmacology* 2004;**47**:892–903.
24. Dean JM, Farrag D, Zahkousk SA, et al. Cerebellar white matter injury following systemic endotoxemia in preterm fetal sheep. *Neuroscience* 2009;**160**:606–615.
25. Szeto HH. Mitochondria-targeted peptide antioxidants: novel neuroprotective agents. *AAPS J* 2006;**8**:E521–E531.
26. Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. *Free Radic Biol Med* 1999;**26**:463–471.
27. Zamzami N, Hirsch T, Dallaporta B, Petit PX, Kroemer G. Mitochondrial implication in accidental and programmed cell death: apoptosis and necrosis. *J Bioenerg Biomembr* 1997;**29**:185–193.
28. Warner DS, Sheng H, Batinic-Haberle I. Oxidants, antioxidants and the ischemic brain. *J Exp Biol* 2004;**207**:3221–3231.
29. Gluckman PD, Guan J, Williams C, et al. Asphyxial brain injury—the role of the IGF system. *Mol Cell Endocrinol* 1998;**140**:95–99.
30. Guan J, Mathai S, Harris AP, et al. Peripheral administration of a novel diketopiperazine, NNZ 2591, prevents brain injury and improves somatosensory-motor function following hypoxia-ischemia in adult rats. *Neuropharmacology* 2007;**53**:749–762.
31. Colbourne F, Sutherland GR, Auer RN. Electron microscopic evidence against apoptosis as the mechanism of neuronal death in global ischemia. *J Neurosci* 1999;**19**:4200–4210.
32. Geddes R, Vannucci RC, Vannucci SJ. Delayed cerebral atrophy following moderate hypoxia-ischemia in the immature rat. *Dev Neurosci* 2001;**23**:180–185.
33. Giulian D, Vaca K, Corpuz M. Brain glia release factors with opposing actions upon neuronal survival. *J Neurosci* 1993;**13**:29–37.
34. Urban JL, Shepard HM, Rothstein JL, Sugarman BJ, Schreiber H. Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc Natl Acad Sci USA* 1986;**83**:5233–5237.
35. Piani D, Frei K, Do KQ, Cuenod M, Fontana A. Murine brain macrophages induced NMDA receptor mediated neurotoxicity *in vitro* by secreting glutamate. *Neurosci Lett* 1991;**133**:159–162.
36. Beilharz EJ, Williams CE, Dragunow M, Sirimanne ES, Gluckman PD. Mechanisms of delayed cell death following hypoxic-ischemic injury in the immature rat: evidence for apoptosis during selective neuronal loss. *Brain Res Mol Brain Res* 1995;**29**:1–14.
37. Guan J, Waldvogel HJ, Faull RL, Gluckman PD, Williams CE. The effects of the N-terminal tripeptide of insulin-like growth factor-1, glycine-proline-glutamate in different regions following hypoxic-ischemic brain injury in adult rats. *Neuroscience* 1999;**89**:649–659.
38. Morikawa E, Moskowitz MA, Huang Z, Yoshida T, Irikura K, Dalkara T. L-arginine infusion promotes nitric oxide-dependent vasodilation, increases regional cerebral blood flow, and reduces infarction volume in the rat. *Stroke* 1994;**25**:429–435.
39. Kozniowska E, Oseka M, Stys T. Effects of endothelium-derived nitric oxide on cerebral circulation during normoxia and hypoxia in the rat. *J Cereb Blood Flow Metab* 1992;**12**:311–317.
40. Yamamoto S, Golanov EV, Berger SB, Reis DJ. Inhibition of nitric oxide synthesis increases focal ischemic infarction in rat. *J Cereb Blood Flow Metab* 1992;**12**:717–726.
41. Tanaka K, Fukuuchi Y, Gomi S. Inhibition of nitric oxide synthesis impairs autoregulation of local cerebral blood flow in the rat. *NeuroReport* 1993;**4**:267–270.
42. Yant LJ, Ran Q, Rao L, et al. The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. *Free Radic Biol Med* 2003;**34**:496–502.
43. Muller FL, Lustgarten MS, Jang Y, Richardson A, Van Remmen H. Trends in oxidative aging theories. *Free Radic Biol Med* 2007;**43**:477–503.
44. Seiler A, Schneider M, Forster H, et al. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab* 2008;**8**:237–248.
45. Ran Q, Gu M, Van Remmen H, Strong R, Roberts JL, Richardson A. Glutathione peroxidase 4 protects cortical neurons from oxidative injury and amyloid toxicity. *J Neurosci Res* 2006;**84**:202–208.