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Reactive species and oxidative stress in optic nerve vulnerable to secondary degeneration

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ABSTRACT

Secondary degeneration contributes substantially to structural and functional deficits following traumatic injury to the CNS. While it has been proposed that oxidative stress is a feature of secondary degeneration, contributing reactive species and resultant oxidized products have not been clearly identified *in vivo*. The study is designed to identify contributors to, and consequences of, oxidative stress in a white matter tract vulnerable to secondary degeneration. Partial dorsal transection of the optic nerve (ON) was used to model secondary degeneration in ventral nerve unaffected by the primary injury. Reactive species were assessed using fluorescent labelling and liquid chromatography/tandem mass spectroscopy (LC/MS/MS). Antioxidant enzymes and oxidized products were semi-quantified immunohistochemically. Mitophagy was assessed by electron microscopy. Fluorescent indicators of reactive oxygen and/or nitrogen species increased at 1, 3 and 7 days after injury, in ventral ON. LC/MS/MS confirmed increases in reactive species linked to infiltrating microglia/macrophages in dorsal ON. Similarly, immunoreactivity for glutathione peroxidase and haem oxygenase-1 increased in ventral ON at 3 and 7 days after injury, respectively. Despite increased antioxidant immunoreactivity, DNA oxidation was evident from 1 day, lipid oxidation at 3 days, and protein nitration at 7 days after injury. Nitrosative and oxidative damage was particularly evident in CC1-positive oligodendrocytes, at times after injury at which structural abnormalities of the Node of Ranvier/paranode complex have been reported. The incidence of mitochondrial autophagic profiles was also significantly increased from 3 days. Despite modest increases in antioxidant enzymes, increased reactive species are accompanied by oxidative and nitrosative damage to DNA, lipid and protein, associated with increasing abnormal mitochondria, which together may contribute to the deficits of secondary degeneration.

Introduction

The pathophysiology of injury to white matter tracts in the central nervous system (CNS) involves both primary and secondary mechanisms. Outside the primary injury area, neurons and glia remain vulnerable to secondary degeneration, with structural changes and delayed cell death resulting in progressive loss of function (Lu et al., 2000; Park et al., 2004; Payne et al., 2012; Tator and Fehlings, 1991). Excess Ca^{2+} flux and oxidative stress have been implicated in *in vitro* studies, as major contributors to damage following injury (Peng and Jou, 2010). However, the biochemical processes and contributors leading to damage and death in nerve tissue vulnerable to secondary degeneration *in vivo* have not been characterized. Partial transection of the optic nerve (ON), involving a precise primary lesion only to dorsal axons, leaves those in ventral ON intact but vulnerable to secondary degeneration (Fitzgerald et al., 2009a; Levkovitch-Verbin et al., 2003). This *in vivo* model allows spatial separation of primary from secondary injury, and the comprehensive assessment of biochemical pathways contributing specifically to secondary degeneration in a white matter tract of the CNS.

Oxidative stress is a hallmark of neurodegenerative diseases such as glaucoma (Izzotti et al., 2006; Tezel, 2006; Uttara et al., 2009) and CNS injury (Carrico et al., 2009; Fitzgerald et al., 2010; Lu et al., 2000; Park et al., 2004; Tator and Fehlings, 1991; Wells et al., 2012) and occurs as a consequence of excess reactive species. Reactive species play a dual role, with both beneficial and harmful effects well documented, as

Abbreviations: AGE, advanced glycation end-product; BSA, bovine serum albumin; CM-H_2DCF, chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate; CML, carboxymethyl lysine; DHE, dihydroethidium; DMSO, dimethyl sulfoxide; GFAP, glial fibrillary acidic protein; GPx1, glutathione peroxidase 1; HNE, 4-hydroxynonenal; HO-1, heme oxygenase-1; LC/MS/MS, liquid chromatography/tandem mass spectroscopy; MPT, mitochondrial permeability transition; NADH/NADPH, nicotinamide adenine dinucleotide/phosphate; 3-NT, 3-nitrotyrosine; 8OHDG, hydroxyguanosine; OMM/IMM, outer/inner mitochondrial membrane; ON, optic nerve; PEG-SOD, polyethylene glycol superoxide dismutase; PFA, paraformaldehyde; ROS, reactive oxygen species; RNS, reactive nitrogen species; TPBN, phenyl-*N*-*tert*-butyl nitrone; VDAC, voltage-dependent anion channel.

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reviewed by Valko et al. (2007). Enzymatic antioxidant defence systems such as glutathione peroxidase (GPx) and manganese superoxide dismutase (MnSOD), together with non-enzymatic antioxidants including ascorbic acid and glutathione, act to maintain redox balance (Valko et al., 2007). However, when there is an abnormal increase in production of reactive oxygen or nitrogen species (ROS/RNS) and/or a decrease in antioxidant activity, antioxidant defences are overwhelmed, resulting in oxidative stress (Kowaltowski et al., 2009). Excess ROS and RNS can oxidise DNA, lipids and proteins to toxic metabolites (Pamplona et al., 2005; Xiong and Hall, 2009). Moreover, an altered redox environment can activate signalling pathways leading to cell damage and death (Valko et al., 2007). Oxidative damage, particularly in the form of lipid oxidation, has been demonstrated following traumatic brain injury (Hall et al., 2010). Increases in a wide spectrum of indicators of oxidative stress provide circumstantial evidence that highly reactive oxidants mediate secondary degeneration following neurotrauma (Cornelius et al., 2013; Halliwell, 2006), and damage in a range of neurological conditions (Dasgupta et al., 2013; Sultana et al., 2006). However, direct measures of the causes and consequences of oxidative stress in white matter exclusively vulnerable to secondary degeneration following neurotrauma are lacking.

ROS consist of radical and non-radical species that can be formed by the partial reduction of oxygen, and include superoxide anion radical ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and the hydroxyl radical ($^{\bullet}OH$) (Camello-Almaraz et al., 2006). RNS also include radical and non-radical species, such as nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$). There is evidence that in ON vulnerable to secondary degeneration, mitochondrial dysfunction and oxidative stress occur early after injury (Cummins et al., 2013; Szymanski et al., 2013). However, the identities of the ROS and/or RNS that contribute, and the nature of oxidative damage that they cause, are not clearly understood. Most ROS are highly reactive molecules, rendering them unstable and difficult to detect in biological samples. Therefore, accurate detection of ROS/RNS requires labelling and/or imaging of live tissue, using particular compounds that react with reactive species and form fluorescent or other specific products. Here, we employ *in vivo* labelling of ROS/RNS, and immunohistochemical detection of antioxidant enzymes and indicators of oxidative damage to proteins, lipids and DNA following partial ON transection. The data generated allow us, for the first time, to develop a picture of the contributors to, and consequences of, oxidative stress in ON vulnerable to secondary degeneration *in vivo*.

Materials and methods

Animals

Procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research, and were approved and monitored by the University of Western Australia Animal Ethics Committee. Adult, female Piebald-Virol-Glaxo (PVG) hooded rats were procured from the Animal Resources Centre (Murdoch, WA), and housed under temperature controlled conditions on a 12-h light/dark cycle, with access to standard rat chow and water *ad libitum*.

Partial ON transection

The partial ON transection procedure was conducted as described previously (Fitzgerald et al., 2010). Briefly, PVG rats were anaesthetized intraperitoneally (i.p.) (50 mg/kg ketamine hydrochloride and 10 mg/kg xylazil hydrochloride, Troy Laboratories, NSW, Australia). Right ONs were surgically exposed by incising the skin overlying the skull and deflecting lachrymal tissue. A diamond keratotomy knife (Geuder, Germany) was used to make a controlled dorsal incision in each ON, to a depth of 200 μm. Post-operative analgesia was administered subcutaneously (2.8 mg/kg carprofen, Norbrook Australia, Pty. Ltd., VIC, Australia). Controls were uninjured normal animals, as we have previously demonstrated no change following sham anaesthesia and surgery (Fitzgerald et al., 2009a). The contralateral ONs were not used as controls, due to demonstrated changes in the opposite eye following ON injury (Bodeutsch et al., 1999) and thus do not provide an appropriate or useful baseline for comparison of oxidative stress changes in our model. All animals were euthanased with Lethabarb© (800–1000 mg/kg i.p., Virbac, Australia Pty. Ltd., NSW, Australia) at 1 h, 1, 3 or 7 days after injury (total $n = 24$/time point).

Detection of reactive species in previously frozen sections

With animals under ketamine-xylazil anaesthesia as described above, right ONs ($n = 6$/time point) were dissected from the ocular cavity, collected onto a microscope slide maintained at −20 °C over a bed of dry ice, mounted in optical cutting temperature compound then snap-frozen in Eppendorf tubes in liquid nitrogen, and stored in airtight zip-lock bags at −80 °C, to avoid desiccation. Tissue was cryosectioned longitudinally at −20 °C and free floating tissue sections (20 μm) from each experimental animal were collected into 30% sucrose in phosphate buffered saline (PBS) in one well of the top row of each of six 24-well trays, to facilitate subsequent labelling and avoid multiple freeze–thaw cycles. Trays containing sections were stored at −80 °C. Labelling and washing solutions were dispensed in rows of the trays and sections were transferred between solutions in droplets, using forceps that did not meet, to avoid damaging the tissue; incubations were timed to ensure equal durations. Sucrose was removed by washing sections in PBS followed by labelling at room temperature, in the dark, in either: 30 μM chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCF) (Cell Biolabs, CA, USA) in PBS for 15 min; 100 μM Amplex UltraRed (Life Technologies, VIC, Australia) in 50 mM sodium citrate buffer (pH 6.5) containing 0.2 U/ml horse radish peroxidase (Life Technologies, VIC, Australia) for 30 min; or 10 μM dihydroethidium (DHE) (Life Technologies, VIC, Australia) in PBS for 10 min. Stock solutions of CM-H_2DCF and DHE were prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich, MO, USA) and stored at −20 °C under argon; Amplex UltraRed was used according to manufacturer's instructions. Some sections were pretreated with 666 U/ml polyethylene glycol superoxide dismutase (PEG-SOD) in PBS for 15 min or 2 mM phenyl-*N*-*tert*-butyl nitrone (TPBN) for 60 min as described (Ma et al., 2009). Tissue sections were washed for 10 min in PBS, and then fixed in 4% paraformaldehyde (PFA, ProSciTech, QLD, Australia) in PBS, for 10 min, washed in PBS, dried, and mounted on glass slides with Fluoromount-G (Southern Biotech, AL, USA). Preliminary studies indicated that exposure of the sections to light (required for imaging) increased fluorescence dramatically within seconds, and that fixation reduced this problem.

In vivo labelling with dihydroethidium (DHE) and detection of reactive species by LC/MS/MS quantification of DHE and its oxidation products

As DHE is only sparingly soluble in aqueous buffers, DHE was bound to bovine serum albumin (BSA, Fraction V-essentially fatty acid free, Sigma-Aldrich, MO, USA) on the day of use as follows. Twenty-five millimolar DHE stock was prepared in argon purged DMSO with vigorous vortexing and minimal exposure to light. The DHE stock was added drop-wise to 7.5 mM BSA in sodium phosphate buffer (50 mM, pH 7.4) such that the final concentrations were DHE 2.5 mM, DMSO 10% and BSA 6.8 mM. Following incubation at 37 °C for 15 min, diethylene triamine pentaacetic acid (Sigma-Aldrich) was added to a final concentration of 0.1 mM and the DHE-BSA preparation was stored on ice, in the dark until use.

Under ketamine-xylazil anaesthesia as described above, the ON partial transection injury sites of animals that had undergone partial ON transection 1 or 7 days previously were re-exposed and the dural sheath around the injury site (both dorsal and ventral) removed. Fifty microliters of DHE-BSA was pipetted directly onto the ON at the injury site, ensuring full immersion of the nerve in labelling solution. ONs were