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Minocycline protects the immature white matter against hyperoxia

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ABSTRACT

Poor neurological outcome in preterm infants is associated with periventricular white matter damage and hypomyelination, often caused by perinatal inflammation, hypoxia–ischemia, and hyperoxia. Minocycline has been demonstrated in animal models to protect the immature brain against inflammation and hypoxia–ischemia by microglial inhibition. Here we studied the effect of minocycline on white matter damage caused by hyperoxia. To mimic the 3- to 4-fold increase of oxygen tension caused by preterm birth, we have used the hyperoxia model in neonatal rats providing 24 h exposure to 4-fold increased oxygen concentration (80% instead of 21% O_2) from P6 to P7. We analyzed whether minocycline prevents activation of microglia and damage of oligodendroglial precursor cell development, and whether acute treatment of hyperoxia-exposed rats with minocycline improves long term white matter integrity.

Minocycline administration during exposure to hyperoxia resulted in decreased apoptotic cell death and in improved proliferation and maturation of oligodendroglial precursor cells (OPC). Minocycline blocked changes in microglial morphology and IL-1β release induced by hyperoxia. In primary microglial cell cultures, minocycline inhibited cytokine release while in mono-cultures of OPCs, it improved survival and proliferation. Long term impairment of white matter diffusivity in MRI/DTI in P30 and P60 animals after neonatal hyperoxia was attenuated by minocycline.

Minocycline protects white matter development against oxygen toxicity through direct protection of oligodendroglia and by microglial inhibition. This study moreover demonstrates long term benefits of minocycline on white matter integrity.

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Introduction

Modern medical care has greatly improved survival of preterm infants, but neurological impairment is still a frequent burden in survivors of premature birth. Poor neurological outcome is often caused by periventricular white matter damage (Volpe, 2009) and has been described to arise from perinatal inflammation, hypocapnia, and hyperoxia (Collins et al., 2001; Leviton and Dammann, 2004; Leviton et al., 2010). Common neurological symptoms of former preterm infants have been shown to be caused by oxygen toxicity in a hyperoxia mouse model, such as hypomyelination and motor hyperactivity (Schmitz et al., 2011, 2012). While brain injury in term infants caused by hypoxia–ischemia ("asphyxia") can nowadays be prevented using hypothermia, interventions to avoid white matter damage in preterm infants are not available. Minocycline is a tetracycline-antibiotic that has been demonstrated to exert neuroprotective properties in various models of brain injury, including hypoxia–ischemia (Arvin et al., 2002; Cai et al., 2006; Tang et al., 2010) and perinatal inflammation/infection (Fan et al., 2005). Beneficial effects of minocycline have so far largely been attributed to inhibition of microglial activation. In adults, minocycline has been shown to improve neurological outcome after stroke (Lampl et al., 2007). In a report on patients suffering from active relapsing–remitting multiple sclerosis, minocycline has been observed to prevent disease deterioration (Zabad et al., 2007). However, recent data indicate that minocycline might act on multiple cell types, i.e. minocycline prevented activation of astroglia in the rat optic nerve after prolonged ischemia (Cai et al., 2010). However, whether minocycline is effective in protecting the immature brain against oxygen toxicity is not known. High arterial oxygen tension in very immature preterm infants has been documented in several clinical

Abbreviations: CC, corpus callosum; EC, external capsule; FA, fractional anisotropy; IL, interleukin; IFN, interferon; iNOS, inducible NO synthase; MHC II, major histocompatibility class II; MBP, myelin basic protein; MRI, magnet resonance imaging; NGS, normal goat serum; Olig2, oligodendrocyte transcription factor 2; OPC, oligodendroglial precursor cell; WM, white matter; WMD, white matter damage; PFA, paraformaldehyde; ROI, region of interest; SOX10, (SRY-related HMG-box) 10; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl-transferased UTP nick end labeling.

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studies to coincide with neurological impairment and periventricular leukomalacia (Collins et al., 2001; Deulofeut et al., 2007; Leviton et al., 2010). Preterm infants experience a several-fold increase of oxygen tension up to 64 or 85 mm Hg, at a developmental period when physiological in utero environment would provide much lower levels of 25 mm Hg (Castillo et al., 2008). This relative hyperoxia has been mimicked in hyperoxia rodent models applying 60 to 80% oxygen for several hours up to several days causing a several fold increase of the physiological oxygen concentration in postnatal rats and mice (Felderhoff-Mueser et al., 2004; Schmitz et al., 2011; Vottier et al., 2011). However, so far, microglial responses of the immature brain to oxygen toxicity are not well defined, and protection by minocycline is untested in oxygen toxicity. In this study with rats, we aimed to clarify if 1) neonatal hyperoxia causes microglial reactions that contribute to white matter damage, 2) minocycline may protect OPCs in a direct way, 3) acute minocycline administration during neonatal exposure to hyperoxia has long term benefits on white matter integrity in the young adult.

Methods

Animals and hyperoxia exposure

All animal experiments were performed in accordance with international guidelines for good laboratory practice and were approved by the animal welfare committees of Berlin, Germany. Six-day-old (P6) Wistar rats were subjected to 24 h hyperoxia (80% O_2) till P7. Litters were divided into the three experimental groups of hyperoxia, hyperoxia with minocycline administration, and control pups. Newborn rats exposed to hyperoxia were placed along with their mothers, in a chamber containing 80% O_2 (OxyCycler, BioSpherix, Lacona, NY, USA). Minocycline was injected i.p. at the beginning of exposure and at 12 h exposure to hyperoxia. The control pups of each litter were kept in room-air with a second lactating mother. During recovery in room air, all pups exposed to hyperoxia were reunited with their biological mother until the age of further experimental use. The pups after hyperoxia with or without minocycline appeared normal and did not suffer weight loss or other morbidities.

Immunofluorescence

Rats at P7, P9, P11, P15 and P30 were anesthetized following animal welfare committee guidelines, and transcardially perfused with PBS and then 4% paraformaldehyde (PFA). Brains were dissected out, post-fixed with 4% PFA overnight at 4 °C. Fixed brains were preserved in 10% glycerol in PBS. For tissue sections, brains were rinsed in 1× PBS, then frozen in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham NC) and mounted on a Microm HM400 microtome (Microm International GmbH, Walldorf, Germany). Sections were cut (10–20 μm) and stored in a 1× PBS, 0.05% sodium azide solution. For immunohistochemistry, sections were blocked at room temperature for at least 1 h in blocking solution [1% bovine serum albumin (BSA), 0.3% Triton X-100, and 20% normal goat serum (NGS) in 1× PBS]. Primary antibodies were diluted using carrier solution (1% BSA, 0.3% Triton X-100 and 1% NGS in 1× PBS). Polyclonal mouse antibody to NG2 chondroitin sulfate proteoglycan (Chemicon, Temecula CA) was diluted 1:500. Monoclonal mouse antibody to myelin basic protein (MBP) (Covance, VA) was diluted 1:500. The monoclonal rabbit Ki67 antibody (Leica Biosystems, Newcastle, UK) was diluted 1:500. Monoclonal mouse CC1 antibody was diluted 1:500 (Abcam, Cambridge MA; Calbiochem, Darmstadt, Germany). Rabbit Iba1 antibody (Wako Chemicals, Richmond VA) was diluted 1:750. Brain sections were incubated in primary antibodies at 4 °C overnight. Rinses were carried out in carrier solution at room temperature, with three changes of solution every 10 min. All secondary antibodies used were from Jackson Immunoresearch Laboratories, West Grove PA, in carrier solution: FITC-conjugated goat anti-mouse IgG (1:200), FITC-conjugated goat anti-rabbit IgG (1:200), CY3/Rhodamine conjugated goat anti-mouse IgG (1:200) and CY3/Rhodamine conjugated goat anti-rabbit IgG (1:200). Incubation was carried out at room temperature for 1 h, followed by three washes as described above. Sections were then stained with DAPI for 10 min and after three washings with PBS mounted with Mowiol.

Cell cultures

Primary mixed glial cultures were prepared from E19 pregnant Sprague–Dawley rats by mechanical dissociation according to the method of McCarthy and de Vellis (1980) as previously described (Gallo and Armstrong, 1995; Schmitz et al., 2011). To obtain microglia cell suspensions, mixed cultures (10–12 days old) were shaken for 2 h, and supernatants with detached microglia were used for microglia culture experiments. To obtain oligodendrocyte progenitor cell (OPC) suspensions, media of the mixed cultures was replaced before overnight shaking of the flasks. To minimize contamination by microglial cells, the detached cell suspension was incubated in succession for 45 min each in 60 mm dishes. OPCs enriched by this method contained >95% GD31 cells labeled by the LB1 monoclonal antibody (Curtis et al., 1988) with <0.5% $GFAP^+$ astrocytes and <0.5% $Ox42^+$ microglia. For Western blot protein analysis, astrocytes, after trypsinization, were plated on 6 well plates in a density of 2×10^5 cells per well in 2 mL DMEM with 10% FCS until confluent. For sub-confluent astrocyte cultures, 1×10^5 cells in 2 mL DMEM containing 10% FCS were transferred to 6 well plates and cultured for 48 h, media was changed the day after trypsinization. The 6 well plates were then used for experiments at either 80% or 21% O_2. OPCs were plated on either poly-lysine-coated 24 well plates at a density of 150,000 cell per well or on poly-lysine-coated 25 mm coverslips kept in 6 well plates at a density of 300,000 cells per well. OPCs were maintained in a chemically defined Dulbecco's Modified Eagle's Medium (Invitrogen Corporation, Carlsbad, CA, USA) containing D-glucose (4 g/L), L-glutamine (4 mM), sodium pyruvate (1 mM), human apo-transferrin (50 μg/mL), bovine pancreatic insulin (5 μg/mL), sodium selenium (30 nM), hydrocortisone (10 nM), D-biotin (10 nM), bovine serum albumin (1 mg/mL), and recombinant human platelet derived growth factor-AA (10 ng/mL). Cells for minocycline experiments were pre-incubated with 10 μM minocycline for 1 h.

Exposure of primary microglia to hyperoxia

Harvested microglia were placed in 6-well plates using 2 mL DMEM growth media with 1% fetal calf serum (FCS, Gibco, Life Technologies, Grand Island NY) for 500,000 microglia per well. All microglia plates were cultured in an incubator (Firma) under 21% O_2, 5% CO_2 and 15% N_2 at 37 °C for the first 24 h. Then, microglia were kept under various conditions: 1) 21% O_2 without minocycline, 2) 21% O_2 with 10 μM minocycline, 3) 80% O_2 without minocycline, and 4) 80% O_2 with 10 μM minocycline. For cytokine release measurements in supernatants, cultures were stopped after 6 h, 12 h, and 24 h of culture time. For RNA analysis, culture duration was 3 h, 6 h, and 24 h. To obtain microglia-conditioned supernatants for OPC culture experiments, media was withdrawn and collected after 24 h of culture time.

Immunocytochemistry

Live staining for cell surface antigens with A2B5 antibodies (Bansal and Pfeiffer, 1989) was performed as previously described (Yuan et al., 1998). Briefly, live cells were incubated at room temperature for 1 h with primary antibodies diluted 1:10 in DMEM, followed by fluorescein-conjugated goat anti-mouse IgM for 45 min. After three washes in PBS, cells were fixed in 4% paraformaldehyde (pH 7.3 in PBS) for 10 min at room temperature and washed in PBS. Coverslips were then mounted in DAPI-Containing Vectashield. For double staining with Ki67, cells after live staining, fixation and washing were blocked

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