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Full-length Article

Oral administration of the nitroxide radical TEMPOL exhibits immunomodulatory and therapeutic properties in multiple sclerosis models

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ABSTRACT

Therapies with both immunomodulatory and neuroprotective properties are thought to have the greatest promise in reducing the severity and progression of multiple sclerosis (MS). Several reactive oxygen (ROS) and reactive nitrogen species (RNS) are implicated in inflammatory-mediated damage to the central nervous system (CNS) in MS and its animal model, experimental autoimmune encephalomyelitis (EAE). TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a stable nitroxide radical with potent antioxidant activity. The goal of our studies was to investigate the immunomodulatory effects and therapeutic potential of orally-delivered TEMPOL in the mouse EAE model. Mice receiving TEMPOL chow *ad libitum* for 2 weeks prior to induction of active EAE showed delayed onset and reduced incidence of disease compared to control-fed animals. Reduced disease severity was associated with limited microglial activation and fewer inflammatory infiltrates. TEMPOL's effects were immunomodulatory, not immunosuppressive: T cells produced less interferon-γ and tumor necrosis factor-α, and TEMPOL-fed mice exhibited a shift towards T_H2-type antibody responses. Both myeloid and myeloid-dendritic cells of TEMPOL-fed EAE animals had significantly lower levels of MHC class II expression than controls; CD40 was also significantly reduced. TEMPOL administration was associated with an enrichment of $CD8^+$ T cell populations and $CD4^+FoxP3^+$ regulatory populations. TEMPOL reduced the severity of clinical disease when administered after the induction of disease, and also after the onset of clinical symptoms. To exclude effects on T cell priming *in vivo*, TEMPOL was tested with the passive transfer of encephalitogenic T cells and was found to reduce the incidence and peak severity of disease. Protection was associated with reduced infiltrates and a relative sparing of neurofilaments and axons. The ability of oral TEMPOL to reduce inflammation and axonal damage and loss demonstrate both anti-inflammatory and protective properties, with significant promise for the treatment of MS and related neurological disorders.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and demyelinating neurodegenerative disorder, characterized pathologically by central nervous system (CNS) alterations in the vasculature, inflammatory infiltrates, demyelination, glial scarring, oligodendrocyte loss, and axonal damage and loss (Frohman et al., 2006). The irreversible axonal loss and neurodegeneration are thought to be the major correlate of chronic disability (Bjartmar and Trapp, 2003) and likely starts early in disease (Ferguson et al., 1997; Trapp et al., 1998). Approved disease modifying therapies (DMTs) target inflammatory processes and fall into two groups: those well tolerated but with partial efficacy, or those with greater efficacy and increased risk profiles (Kieseier et al., 2011; Stuve, 2009). Development of orally efficacious, safe, and well-tolerated therapeutics with immunomodulatory and neuroprotective properties remains a priority.

Abbreviations: CFA, complete Freunds adjuvant; EAE, experimental autoimmune encephalomyelitis; MOG, Myelin Oligodendroglial Protein; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl.

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In experimental autoimmune encephalomyelitis (EAE), the mouse model of MS, autoreactive T cells that produce pro-inflammatory cytokines including tumor necrosis factor α (TNFα), interferon-γ (IFNγ), interleukin (IL)-17, and granulocyte macrophage colony stimulating factor (GM-CSF), travel to the CNS to cause disease (Sospedra and Martin, 2005). Both CD4 and CD8 T cells may contribute directly to pathogenesis via direct contact, but phenotypical and molecular profiles vary and define pathogenic or suppressive/regulatory roles (Hauser et al., 2008; Johnson et al., 2010; Racke, 2009). While demyelination and associated axonal loss appear secondary to inflammation (Trapp et al., 1998), neurodegeneration seen prior to inflammatory cell infiltration in EAE and the progressive loss of function after the inflammatory phase has subsided (Hobom et al., 2004; Qi et al., 2006) seem distinct. Recent findings implicate mitochondrial dysfunction secondary to energy imbalance and increases in reactive oxygen species (ROS) as sources of oxidative damage not directly attributable to inflammation (Mahad et al., 2015, 2009; Su et al., 2009).

Emerging evidence implicates ROS and reactive nitrogen species (RNS), focusing on superoxide, nitric oxide, and their intermediates, as contributors to several mechanisms underlying the pathogenesis of MS and EAE (Gilgun-Sherki et al., 2004; Gonsette, 2008a). ROS drive morphological alterations that promote leukocyte traffic across the blood-brain barrier (BBB) (Schreibelt et al., 2006; Van der Goes et al., 2001). Infiltrated leukocytes produce ROS that induce myelin phagocytosis and, therein, myelin breakdown by macrophages, oligodendrocyte damage, as well as axonal and neuronal injury and loss (Smith et al., 1999, 2001; van Meeteren et al., 2004). Microglia and neurons generate peroxynitrite, a principal mediator of the oxidative stress and excitotoxicity that drives neurodegenerative processes in MS (Gonsette, 2008b; Torreilles et al., 1999).

Nitroxide radicals exhibit antioxidant activity and have been reported to react with both ROS and RNS, directing a shift from their use as biophysical tools to potential therapeutics (Soule et al., 2007b). Nitroxides are flexible, cycling via redox transformation between the oxidation states of nitroxide radical, hydroxylamine, and the oxoammonium cation (Fig. 1A) (Soule et al., 2007a), as they interact with biological oxidants and reductants. TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) is a small (MW 172 Da) stable nitroxide radical that can readily permeate biological membranes (Bonini et al., 2002), allowing for significant tissue and intracellular accumulation. Indeed, TEMPOL exerts beneficial effects in animal models of shock, hypertension, diabetes, ischemia-reperfusion injury, spinal cord injury, traumatic brain injury (Deng-Bryant et al., 2008; Kato et al., 2003;

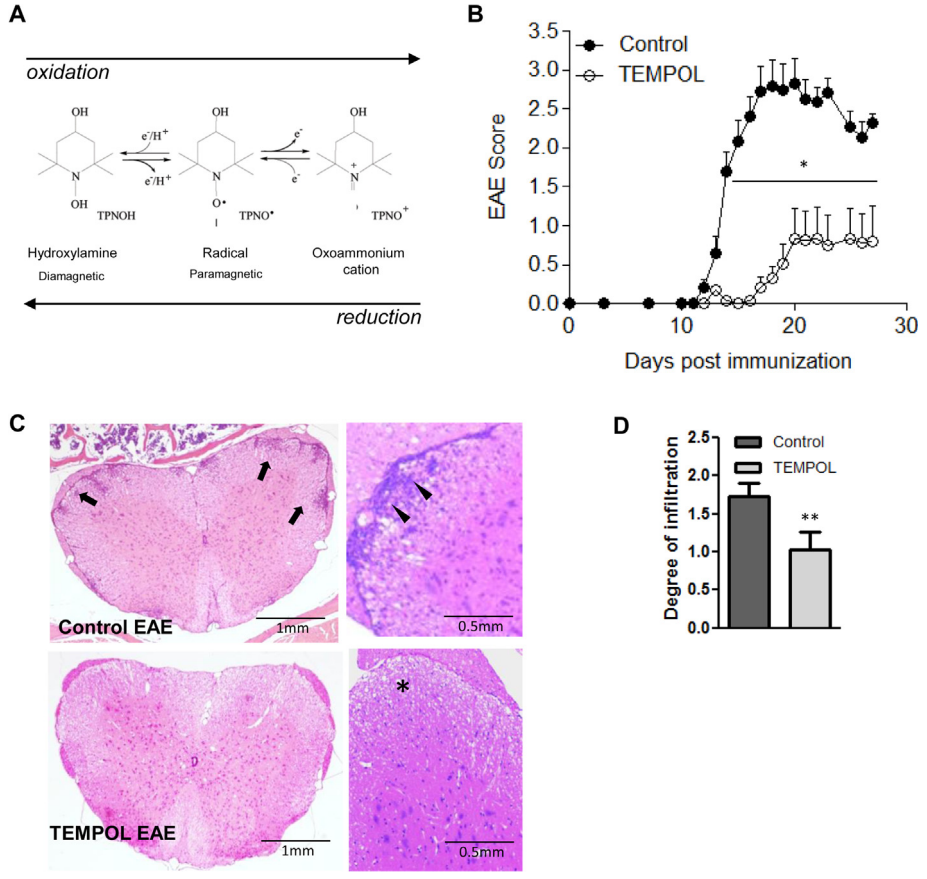

Fig. 1. The nitroxide compound TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl) given orally reduces clinical disease in EAE. The five- or six-membered rings (Fig. 1A) contain a nitrogen atom – the molecule bound to the nitrogen dictates the various properties of the compound. Conversion of nitroxide radical to hydroxylamine or oxoammonium cation occurring *in vivo* are shown. Nitroxides exist *in vivo* in equilibrium between the nitroxide radical form [detected by electron paramagnetic resonance studies (EPR) and the reduced "hydroxylamine" form (not detected by EPR)]. Fig. 1B. C57BL/6J animals maintained on TEMPOL chow (•) for 2 weeks prior to induction of EAE show resistance to the induction of active EAE compared to animals on control chow (○). $n = 15$ animals/group, 4/15 TEMPOL-fed animals vs. 14/15 control fed animals presented with a limp tail or greater during the course of the experiment. TEMPOL-fed animals are resistant to induction of chronic EAE as shown by reduced incidence and overall disease burden. One representative of four experiments with similar results is shown; $p < 0.01$ unpaired two-tailed T test comparing daily disease scores of control to TEMPOL-fed animals. Inflammatory infiltrates and axonal loss are reduced in mice fed TEMPOL prophylactically. In an experiment with similar results to Fig. 1B, tissues were taken at day 21 for histological analyses (Fig. 1C). Control-fed animals show significant infiltrates stemming from the meninges and infiltrating the parenchyma of the white matter (arrows). (D) The degree of leukocyte infiltration in TEMPOL-fed mice was significantly lower than in controls. $n = 5$ mice per treatment group were analyzed, each with 8–10 levels spread over the entire cord examined per mouse. $^{**}p = 0.002$ Mann-Whitney Rank Sum test.