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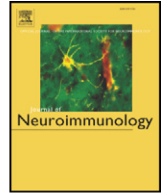

The antioxidant idebenone fails to prevent or attenuate chronic experimental autoimmune encephalomyelitis in the mouse

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ABSTRACT

Oxidative stress and mitochondrial dysfunction appear to contribute to neurodegenerative processes during multiple sclerosis (MS). Thus, antioxidants may represent a therapeutic option for MS. The antioxidant idebenone was proven to be beneficial in Friedreich's ataxia and Leber's hereditary optic neuropathy, two disorders caused by mitochondrial alterations. Here we showed that idebenone protected neuronal HT22 cells from glutamate-induced death in vitro. However, in experimental autoimmune encephalomyelitis, idebenone failed to affect disease incidence or onset when applied preventively, or to reduce disease severity when applied therapeutically. Histopathological examination of CNS from idebenone treated mice showed no improvement in inflammation, demyelination, or axonal damage. Thus, we hypothesize that idebenone treatment will likely not benefit patients with MS.

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1. Introduction

Multiple sclerosis (MS) is an immunological disease (Sawcer et al., 2011) in which an immune response appears to be directed towards myelin-related auto-antigens. Autoreactive T cells are believed to orchestrate an immune reaction leading to damage of myelin and oligodendrocytes and thus severe disability in young adults (Infante-Duarte et al., 2008). During the last decades, studies in MS and the animal model experimental autoimmune encephalomyelitis (EAE) have demonstrated that in addition to myelin and oligodendrocyte damage, axonal and neuronal injury are implicated in disease severity (Aktas et al., 2005; Vogt et al., 2009). In this context, mitochondrial injury seems to link inflammation and axonal neurodegeneration (Lassmann and van Horssen, 2011). It has been recently shown that intra-axonal mitochondrial damage is an early sign of neurodegeneration that precedes and contributes to focal and reversible alterations in axon morphology. Mitochondrial pathology and subsequent focal axonal injury appears to be triggered by macrophage-derived reactive oxygen and nitrogen species and to be independent of demyelinating processes (Nikic et al., 2011). Free radicals may affect ATP production by altering the respiratory chain and inducing the production of the highly toxic peroxynitrite (ONOO) (Brown and Borutaite, 2004), which lead to degradation of cytoskeletal and membrane proteins and the release of the apoptotic mediator cytochrome C (Gao, 2010). Thus, therapies targeting the generation of free radicals emerge as a promising approach for diseases with mitochondrial involvement such as MS.

Idebenone, 6-(10-hydroxydecyl)-2,3-dimethoxy-5-methyl-l,4-benzoquinone, is a synthetic analog of coenzyme Q_{10}, a cell membrane antioxidant and essential constituent of the ATP-producing mitochondrial electron transport chain. In contrast to Q_{10}, the synthetic idebenone has increased bioavailability due to its shortened hydrophobic tail (Geromel et al., 2002) while maintaining its anti-oxidant properties. Idebenone is capable of restoring the electron flow in the mitochondrial electron transport chain (Geromel et al., 2002) and has been shown to protect against cerebral ischemia and prevent nerve damage in the CNS (Rego et al., 1999; Di Prospero et al., 2007). Apart from its anti-oxidant properties, idebenone exerts anti-inflammatory effects in vitro (Civenni et al., 1999). Taking these properties into consideration, we hypothesized that idebenone may represent a therapeutic option for progressive MS, a disease form in which current immunomodulatory treatments are not sufficiently effective (Stadelmann, 2011).

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To investigate the therapeutic potential of idebenone in chronic neuroinflammation, we applied idebenone orally to EAE mice using both a preventive and a therapeutic treatment strategy.

2. Materials and Methods

2.1. Neuroprotection assay

For the assessment of neuroprotective capacities of idebenone in vitro, hippocampal HT22 cells were seeded at 5000 cells per well in 96 well plates and cultured in 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine, and 0.35% glucose for 24 h. On the following day, the cells received fresh medium and were incubated with varying concentrations of idebenone in 1% DMSO or vehicle control for 2 h before incubation with 5 mM or 10 mM glutamate (Sigma, G1626). Cell viability was measured using the crystal violet assay as previously described (Dörr et al., 2002; Herges et al., 2011). Briefly, the cells were stained for 30 min with 0.5% crystal violet in 20% methanol. After washing and drying overnight, crystal violet was dissolved in 50 ml/well of 0.1 M sodium citrate solution diluted in 50% ethanol and subsequently quantified photometrically by absorbance at 600 nm in a micro plate reader (Promega Glomax). Values were expressed as the percentage of survival compared with untreated controls.

2.2. EAE induction and treatment

Female 6–8 week old C57BL/6 mice were immunized subcutaneously with 200 μg $MOG_{35–55}$ peptide (purity > 95%, Pepceuticals, Leicester, UK) emulsified in an equal volume of PBS and complete Freund's adjuvant containing 6 mg/ml Mycobacterium tuberculosis H37Ra (Difco, FranklinLakes, NJ). *Bordetella pertussis* toxin (200 ng, PTX, List Biological Laboratories, Campbell, CA) was administered intra-peritoneally at days 0 and 2 post-immunization. The mice were weighed and scored daily as follows: 0 = no disease; 1 = complete tail paralysis; 2 = hindlimb paresis; 3 = hindlimb plegia; 4 = paraplegia and forelimb weakness; 5 = moribund or death due to EAE. 100 mg/kg body weight idebenone (≈2 mg/mouse; Santhera Pharmaceuticals, Switzerland) was resuspended in 0.5% carboxymethyl cellulose (CMC) and applied daily by oral gavage. For preventive treatment, 100 mg/kg idebenone or vehicle (0.5% CMC) was administered daily starting at day 7 after immunization. Therapeutic treatment with 100 mg/kg idebenone or vehicle was started when animals reached a clinical score of 1 (complete tail paralysis).

All experimental procedures were approved by the regional animal study committee of Berlin (Landesamt für Gesundheit und Soziales Berlin, approval ID G0206/11). All animal work was conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.3. Tissue processing and staining

After terminal anesthesia with xylene/ketamine, mice were transcardially perfused with 100 ml PBS followed by 100 ml of 4% paraformaldehyde (PFA). Tissues were extracted and post fixed overnight in a 4% PFA solution. Tissues were then cryoprotected by overnight incubation at 4 °C in 15%, then 30% sucrose in PBS. Spinal cords were bisected into 8 segments, and embedded together in O.C.T. (Tissue Tek, Sakura), then frozen in methlylbutane with dry ice. The tissue was cut into 12 μm transverse sections on a cryostat. To assess inflammation, tissue sections were stained with hematoxylin and eosin (H&E) according to standard procedures. Demyelination was assessed by luxol fast blue (LFB) staining, and axonal damage assessed by Bielschowsky silver staining, according to standard protocols. Semiquantitative assessment of inflammation was done by counting the number of quadrants of each of the eight transverse segments that contained mild (score = 1) or severe (score = 2) inflammatory pathology. Duplicate sections for each mouse were scored by two independent observers blinded to treatment group, and data are presented as percentage of total affected spinal cord quadrants. Demyelination and axonal damage were assessed in a similar manner, by counting the number of quadrants of transverse spinal cord sections that showed pathological changes, by two observers blinded to treatment group. Pathology was indicated by loss of blue color in LFB stained sections (demyelination) or loss of brown color in Bielschowsky stained sections (axonal loss). Pictures were taken with a Zeiss Observer Z1, AxioCam ICc 1.

2.4. Detection of idebenone in serum and CSF of treated mice

Idebenone concentrations were measured by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Idebenone measurements were performed in a separate cohort of twelve EAE mice that were treated orally for 10 days (100 mg/kg) starting at day 7 post-immunization and sacrificed 30 min after the last administration, by lethal injection of anesthetic, as above. This time point was selected taking into consideration the pharmacokinetic data on idebenone published recently by Heitz et al. (2012). The thoracic cavity was exposed, and the vena cava cut. Blood was collected and then centrifuged for 10 min at 14,000 rpm. The blood serum was removed and stored at −80 °C until further analysis. The mice were then perfused transcardially with 20 ml of PBS, and the musculature was dissected to reveal the dura covering the cisterna magna, for CSF collection. A fine glass capillary tube was inserted through the dura, and CSF was aspirated. Samples visibly contaminated with blood were discarded. The CSF was then stored at −80 °C until further analysis.

For serum, the sample volume of 100 μl was combined with 200 μl of the precipitation reagent (0.01 mg/ml trimipramine-d3 in methanol). For CSF, the sample volume of 15 μl was combined with 15 μl of plasma and 60 μl of the precipitation reagent. The mixtures were centrifuged for 5 min at 16,000 g and 100 μl (serum) or 70 μl (CSF) of the supernatant were transferred into a 1.5 ml-amber glass vial. The chromatographic separation was achieved within 6 min on a Kinetex C18 (100 × 3.0 mm, 2.6 μm) from Phenomenex (Aschaffenburg, Germany) using a gradient consisting of a mixture of solvent A (0.1% HAc with 5 mM NH_4acetate:methanol (90:10)) and solvent B (methanol) pumped at a flow rate of 0.30 ml/min (60 °C). Injection volume was 25 μl. The compounds were detected using a 5500 QTrap® from AB Sciex (Darmstadt, Germany) in positive-ion mode and quantified by multiple-reaction monitoring following two mass transitions for idebenone (m/z): 339.1→196.9 and 339.1→153.7; the internal standard was trimipramine-D3 (m/z): 298.1→102.9. HPLC and the mass spectrometer were controlled by Analyst® Software, version 1.5.1 (AB Sciex, Darmstadt, Germany). Accuracy of the method was verified, as the percent differences between the theoretical value and the experimental value for the controls were less than 7% (bias). A concentration of 2 ng/ml or 1 ng/ml was established as the lower limit of detection of idebenone in serum and CSF, respectively.

2.5. Statistics

The neuroprotection assay was repeated at least three times, and was analyzed by 2-way ANOVA with the Bonferroni correction for multiple comparisons. EAE incidence and time to disease onset was analyzed using the chi-squared test and the log rank test, respectively. EAE clinical scores were analyzed by the Mann–Whitney *U* test. For comparison of the cumulative disease activity, the area under the curve (AUC) from the clinical score plot for each individual mouse was calculated and analyzed with the Mann–Whitney test. p-values < 0.05 were considered significant. GraphPad Prism version 5.01 (GraphPad Software, San DiegoCA) was used for the analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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