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Original Contribution

Biliverdin reductase, a major physiologic cytoprotectant, suppresses experimental autoimmune encephalomyelitis

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Abstract

Oxidative stress plays an important role in the pathogenesis of multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). Bilirubin is regarded today as a potent antioxidant. Recent studies show that the potent antioxidant actions of bilirubin reflect an amplification mechanism whereby biliverdin reductase (BVR) physiologically regenerates bilirubin in a catalytic cycle. We hypothesized that BVR might prove to be a new effective target for the treatment of free radical-mediated diseases. In this study, we demonstrated that treatment with BVR ameliorated both clinical and pathological signs of EAE more efficiently than treatments with traditional antioxidant enzymes. In vitro, interference with cellular BVR activity by siRNA elicited greater increases in reactive oxygen species and cell death than interference with the activities of other antioxidant enzymes. Further studies showed that BVR surpasses other enzymes by the multifactorial functions of its only end product, bilirubin, including anti-complement activity, and an activity that inhibits antibody-dependent cell-mediated cytotoxicity of lymphocytes. Since BVR regenerates bilirubin in a redox cycle without significantly increasing the concentration of bilirubin, our results suggest that BVR may represent a novel strategy for the treatment of multiple sclerosis and other oxidative stress-mediated diseases.

Keywords: Biliverdin reductase; Bilirubin; Heme oxygenase; Experimental autoimmune encephalomyelitis; Multiple sclerosis; Oxidative stress; Reactive oxygen species; Free radical

Introduction

Multiple sclerosis (MS) is believed to be an autoimmune inflammatory demyelinating disease of the central nervous system (CNS) [1]. Despite intensive research, its etiology still remains elusive, and up to now no definitive therapy is available for this disease. In recent years, increasing evidence suggests that oxidative stress plays an important role in the pathogenesis of MS, and that it contributes directly to CNS damage [2,3]. It has been shown that CNS cells, notably oligodendrocytes and neurons, are highly vulnerable to oxidative damage due to many risk factors, including their active oxidative metabolism, high iron content, and relatively low levels of antioxidant defenses [2,4]. Free radicals are produced massively in MS [2]. Their consequence, the oxidative damage to membrane lipids, proteins, and DNA of cells, has been demonstrated in MS lesions [5–7]. Similarly, oxidative injury is important in the development of many other diseases, such as inflammation, ischemic stroke, Parkinson's disease, Alzheimer's disease, and Huntington's disease [8–10]. Accumulating data indicate that treatment with powerful antioxidants might represent an effective therapy for a variety of human disorders, including MS, to reduce disease progression and improve clinical outcome.

In recent years, bilirubin has been demonstrated to be a potent antioxidant substance in vitro and a very effective

Abbreviations: EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; CNS, central nervous system; BVR, biliverdin reductase; GSH, glutathione; HO, heme oxygenase; ROS, reactive oxygen species; RNAi, RNA interference; ADCC, antibody-dependent cell-mediated cytotoxicity; MBP, myelin basic protein; PBS, phosphate-buffered saline; DAI, days after immunization; SOD, superoxide dismutase; DDC, diethyldithiocarbamate; BSO, L-buthionine-sulfoximine; 3-AT, 3-amino-1,2,4-triazole; SnMP, tin-mesoporphyrin; H_2DCF, 2′,7′-dichlorodihydrofluorescein diacetate.

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physiological antioxidant in vivo. It is the final product of heme catabolism as heme oxygenase (HO) cleaves heme to form biliverdin, which is subsequently reduced by biliverdin reductase (BVR) to bilirubin [11,12]. Bilirubin and its serum albumin complex are superoxide scavengers and peroxyl radical-trapping antioxidants [13,14]. Bilirubin suppresses oxidation more strongly than many other antioxidants, including α-tocopherol, ascorbic acid, and catalase, especially under pathological conditions [13,15]. As little as 10 nM bilirubin can protect against almost 10,000-fold higher concentrations of H_2O_2 [16]. Recent studies show that the potent physiologic antioxidant actions of bilirubin reflect an amplification cycle whereby bilirubin, acting as an antioxidant, is itself oxidized to biliverdin and then recycled back to bilirubin by BVR [17]. Since BVR physiologically regenerates bilirubin in a catalytic cycle, and bilirubin represents one of the most abundant endogenous antioxidants in mammalian serum and tissues, we hypothesized that BVR might serve as a new useful pharmacological target for the treatment of oxidative stress-mediated diseases. Here, in experimental autoimmune encephalomyelitis (EAE), one of the most useful models for MS, we demonstrate that treatment with BVR suppresses clinical and pathological signs more efficiently than treatments with traditional antioxidant enzymes. Furthermore, we show that BVR surpasses other antioxidant enzymes by the multifactorial functions of its only end product, bilirubin.

Materials and methods

Induction of EAE and histological studies

Male Lewis rats with body weights around 200 g were purchased from Charles River Laboratories (Laval, Canada). All studies were approved by the Animal Care Committee of the University of British Columbia. EAE was induced in Lewis rats by a single subcutaneous injection in the abdomen with 50 μg guinea pig myelin basic protein (MBP) (Sigma, Saint Louis, MO) emulsified in 100 μl complete Freund's adjuvant (Sigma) containing 10 mg/ml heat-inactivated Mycobacterium tuberculosis H37Ra (Difco, Detroit, MI). The rats were monitored daily after immunization. Clinical EAE was scored in a double-blind fashion as follows: 0, normal; 1, tail limpness; 2, hind limb paraparesis with clumsy gait; 3, hind limb paralysis; 4, tetraplegia; 5, moribund. For histological studies, rats were euthanized and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The lumbosacral spinal cords were immediately removed, embedded in Tissue-Tek, and frozen with 2-methylbutane at −80°C. Serial sections were cut at 10 μm on a Reichert-Jung 2800 Frigocut cryostat. The sections were stained with hematoxylin-eosin for histopathological examination. The severity of inflammation was graded in a double-blind fashion as follows: 0, no inflammation; 1, mild meningeal inflammation and/or rare parenchymal infiltration; 2, moderate meningitis, submeningeal infiltration, and small scattered perivascular infiltration; 3, severe meningitis, parenchymal infiltration, and/or multiple perivascular infiltration; 4, foci of necrosis and/or neutrophilic infiltration [18]. Immunostaining was performed following the established protocol [19]. Rabbit anti-BVR (StressGen, Victoria, Canada) and rabbit anti-bilirubin (Cocalico, Reamstown, PA) were used at 1:500 dilution. An antibody against 8-isoporstane (Oxford Biomedical Research, Oxford, MI) at 1:400 was used for analyses of oxidative damage in lesions. For double-immunostaining, sections were incubated first with anti-BVR followed by antibodies against macrophage differentiation antigen ED1 (Accurate, Westbury, NY), astrocyte antigen GFAP (Accurate), or neurofilaments (Sternberger, Lutherville, MD).

Intrathecal injection and treatment regimen

Four-week osmotic minipumps (Alzet, Cupertino, CA) were each filled with 200 μl artificial cerebrospinal fluid (aCSF) supplemented with 20 μg gentamicin. The pumps were connected to a 5-cm PE-10 polyethylene tube (ReCathCo, Allison Park, PA). To implant the pumps, a small opening was made in the atlanto-occipital membrane overlying the cisterna magna, and the 5-cm tube was inserted caudally into the subarachnoid space. The minipumps were then embedded subcutaneously between the scapulae. On the fifth day after surgery, animals were immunized with MBP. From 8 to 12 days after immunization (DAI), freshly prepared antioxidant enzymes were delivered intrathecally once daily through the polyethylene tube using a Hamilton microsyringe. The minipump was then reconnected to keep the tube clear. Three groups of rats were treated with BVR (StressGen) at different doses that, from low to high, were 2.5, 10, and 40 μg/day, respectively. Animals were treated with SOD (Sigma), catalase (Sigma), glutathione (GSH) reductase (Sigma), or HO-1 (StressGen) with the same regime at increasing doses until maximal effects were achieved. All the antioxidant enzymes were dissolved in aCSF supplemented with 80 μg/100 μl rat albumin and 10 μg/100 μl gentamicin. For control injections, the solution was the same, only without the enzymatic treatment. Since BVR from StressGen was prepared in TE buffer (0.01 M Tris-HCl, 1 mM EDTA), another group of rats were treated with TE buffer as a control.

Cell culture and viability measurements

SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA) were cultured in DMEM (Invitrogen, Ontario, Canada) supplemented with 10% fetal bovine serum and glutamine. Cells were plated into 24-well plates at a density of 50,000 cells/well. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After the indicated treatments, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) colorimetric assay.

RNA interference and antioxidant enzyme activity inhibition

RNA interference (RNAi) of the BVR transcript was performed as described in earlier work [20]. Briefly, small