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Research Paper

Ablation of ferroptosis regulator glutathione peroxidase 4 in forebrain neurons promotes cognitive impairment and neurodegeneration

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ABSTRACT

Synaptic loss and neuron death are the underlying cause of neurodegenerative diseases such as Alzheimer's disease (AD); however, the modalities of cell death in those diseases remain unclear. Ferroptosis, a newly identified oxidative cell death mechanism triggered by massive lipid peroxidation, is implicated in the degeneration of neurons populations such as spinal motor neurons and midbrain neurons. Here, we investigated whether neurons in forebrain regions (cerebral cortex and hippocampus) that are severely afflicted in AD patients might be vulnerable to ferroptosis. To this end, we generated Gpx4BIKO mouse, a mouse model with conditional deletion in forebrain neurons of glutathione peroxidase 4 (Gpx4), a key regulator of ferroptosis, and showed that treatment with tamoxifen led to deletion of Gpx4 primarily in forebrain neurons of adult Gpx4BIKO mice. Starting at 12 weeks after tamoxifen treatment, Gpx4BIKO mice exhibited significant deficits in spatial learning and memory function versus Control mice as determined by the Morris water maze task. Further examinations revealed that the cognitively impaired Gpx4BIKO mice exhibited hippocampal neurodegeneration. Notably, markers associated with ferroptosis, such as elevated lipid peroxidation, ERK activation and augmented neuroinflammation, were observed in Gpx4BIKO mice. We also showed that Gpx4BIKO mice fed a diet deficient in vitamin E, a lipid soluble antioxidant with anti-ferroptosis activity, had an expedited rate of hippocampal neurodegeneration and behavior dysfunction, and that treatment with a small-molecule ferroptosis inhibitor ameliorated neurodegeneration in those mice. Taken together, our results indicate that forebrain neurons are susceptible to ferroptosis, suggesting that ferroptosis may be an important neurodegenerative mechanism in diseases such as AD.

1. Introduction

Aging is the biggest risk factor for neurodegeneration diseases such as Alzheimer's disease (AD); therefore, as the population ages, the incidence of AD increases exponentially. The underlying cause of AD is the degeneration of neuron populations important for learning and memory. The degeneration process includes the progressive degradation of synapses and eventual death of neurons. At present, the modalities of neuron death remain unclear. Because studies have detected signatures of apoptosis in post-mortem brain tissue from AD patients and showed that Aβ could induce apoptosis in neurons, it is thought that apoptosis is likely responsible for the immense neurodegeneration in AD [1,2]. However, concomitant pathological features in AD brains such as augmented inflammation and the chronic nature of the degenerating process cannot be explained by apoptotic cell death alone [3,4]. Further, drugs targeting apoptosis for the treatment of neurodegenerative diseases (e.g., caspase inhibition or MKL inhibition) have been largely ineffective in the clinic [5,6], highlighting the potential for alternative cell death modalities in neurodegeneration.

Oxidative damage is well-demonstrated in AD brains [7,8]. Because the brain is rich in lipids containing polyunsaturated fatty acids (PUFAs), lipid peroxidation is the prominent type of oxidative damage. Indeed, lipid peroxidation is believed to be an early event in AD pathogenesis [9]. Dysregulation of iron, which can augment the production of reactive oxygen species (ROS), is also evident in AD

Abbreviations: AD, Alzheimer's disease; Gpx4, glutathione peroxidase 4; TAM, tamoxifen; CC, cerebral cortex; HC, hippocampus; 4-HNE, 4-hydroxynonenol; GFAP, glial fibrillary acidic protein; Iba-1, ionized calcium-binding adapter molecule-1; Syn, synaptophysin; PSD95, postsynaptic density protein; SNAP25, synaptosome associated protein; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6; IL-1β, interleukin-1 beta; PUFA, polyunsaturated fatty acid

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brains [10]. Interestingly, lipid peroxidation and iron dysregulation identify a new cell death mechanism called ferroptosis, a cell death pathway that is genetically, morphologically, and biochemically distinct from apoptosis [11]. Cells dying through ferroptosis release damage-associated molecular patterns (DAMPs) and lipid metabolites that are immunogenic [12,13], and are predicted to result in elevated inflammation. In fact, inflammation is a pronounced pathological feature of AD [14], which is not a feature of apoptotic cell death. Thus, ferroptosis presents an intriguing modality of neurodegeneration in AD because of its dependence on lipid peroxidation and iron dysregulation as well as its ability to induce inflammation, all of which are signatures of AD.

Lipid peroxidation is the driving force of cell death in ferroptosis [15]. Glutathione peroxidase 4 (Gpx4) is a selenoprotein glutathione peroxidase that can detoxify hydroperoxides in membrane lipids directly, thereby reducing damage to membrane function and preventing the generation of lipid peroxidation-derived reactive products such as 4-hydroxynonenal (4-HNE) [16]. Yang et al. identified Gpx4 as a key regulator of ferroptosis in cancer cells [17]. Subsequently, it was shown that ablation of Gpx4 induces ferroptosis in cell types such as renal tubular cells and T lymphocytes [13,18]. We previously reported that conditional ablation of Gpx4 in neurons of adult mice triggers rapid degeneration of spinal motor neurons likely through ferroptosis [19]. However, because those mice succumbed to motor neuron disease so quickly, it was impossible to discern whether neuron populations important for cognition are vulnerable to ferroptosis. In this study, we sought to investigate the ferroptotic potential of neurons in the forebrain regions of hippocampus and cerebral cortex that are essential for learning and memory and are severely afflicted in AD patients. We generated a novel forebrain neuron specific, tamoxifen inducible Gpx4 knockout mouse model. Following tamoxifen treatment to ablate Gpx4, we observed cognitive impairment and hippocampal neurodegeneration in those forebrain-specific Gpx4 knockout mice. In addition, we detected several markers associated with ferroptosis in those mice. We also showed that neurodegeneration and behavior dysfunction was exacerbated by placing the animals on a diet deficient in vitamin E, a lipid soluble antioxidant with anti-ferroptosis activity. We further showed that treatment with a small-molecule ferroptosis inhibitor attenuated neurodegeneration in those mice. Our results suggest ferroptosis is a potential neurodegenerative mechanism affecting neurons important for learning and memory.

2. Materials and methods

2.1. Animals, diets, and treatments

Gpx4(f/f) mice were previously described [20]. Camk2α-CreER(T2) mice, which express a tamoxifen-activatable form of Cre recombinase in forebrain neurons under the direction of Camk2α promoter [21], were purchased from the Jackson Laboratories (Cat# 012362, Bar Harbor, ME). Gpx4(f/f) mice were bred with Camk2α-CreER(T2) mice to produce Gpx4(f/+);Camk2α-CreER(T2)$^{+/0}$ mice, which were then bred with Gpx4(f/f) mice to produce Gpx4(f/f);Camk2α-CreERT$^{+/0}$ mice, also known as Gpx4BIKO mice. Gpx4BIKO mice [Gpx4(f/f);Camk2α-CreER(T2)$^{+/0}$] were bred with Gpx4(f/f) mice to produce Gpx4BIKO mice and Gpx4(f/f) mice used in the experiments. Gpx4BIKO mice and Gpx4(f/f) littermates at 2–4 months of age were enrolled in experimental cohorts. All cohorts consisted of roughly half males and half females.

To induce Gpx4 ablation, tamoxifen (T5648, Sigma) was dissolved in corn oil (10 mg/ml) and administered by intraperitoneal (i.p.) injection to Gpx4BIKO mice at 60 mg/kg once per day for 5 days. The same treatment also was applied to control Gpx4(f/f) mice.

The vitamin E deficient diet, an AIN-93G-modified rodent diet that lacks vitamin E, was formulated and manufactured by Bio-Serv (Flemington, NJ). To determine the effect of vitamin E deficiency on neurodegeneration, Gpx4BIKO mice and control Gpx4(f/f) mice were fed the vitamin E deficient diet starting at the age of 1 month. Tamoxifen was administered 6 weeks later to ablate Gpx4 in Gpx4BIKO mice.

Liproxstatin-1, a small-molecule ferroptosis inhibitor, was purchased from Selleckchem.com (Cat #-S7699). To evaluate the effect of ferroptosis inhibition on neurodegeneration, after being fed the vitamin E deficient diet for 6 weeks, Gpx4BIKO mice were treated with tamoxifen to ablate Gpx4, as described above. Two days into tamoxifen treatment, liproxstatin-1, which was dissolved in DMSO and then diluted in PBS, was given to one half of the mice via i.p. injection every two days at a dose of 10 mg/kg, whereas the other half of mice received i.p. injection of the vehicle (1.5% DMSO in PBS) only.

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committees of the University of Texas Health Science Center at San Antonio and the Audie Murphy Memorial Veterans Hospital, South Texas Veterans Health Care System.

2.2. Detection of cre-mediated Gpx4 ablation

A PCR-based method was used to detect Cre-mediated recombination of the floxed Gpx4 allele in genomic DNA isolated from tissues. PCR reactions were performed using primers (P1, 5′-TAC TGC AAC AGC TCC GAG TTC-3′; P2, 5′-CTT CAC CAC GCA GCC GTT CT-3′) that would produce a 700 bp-amplicon from the recombined Gpx4 (rGpx4) allele.

2.3. Behavior tasks

Spatial learning and memory function was assessed by the Morris Water Maze task as described [22]. In brief, mice were trained to find a hidden platform in opaque water for 5 days with 4 acquisition-trials per day from pseudorandomized start positions. Escape latency, or time to find the hidden platform, was recorded as an index of spatial learning and memory (60 s max). Probe trials (where the submerged platform was removed) were performed on day 6 after acquisition trials whereby total time spent searching in the area previously containing the platform was calculated as an additional memory metric (30 s max). Average acquisition-trial time (s) divided by distance traveled during that time (m) was calculated as a proxy for active participation in the task or what we call the motivational quotient (MQ). Significant deviations from the average MQ per group, declared as non-swimmers, were determined using the modified Thompsons tau technique for outlier detection and removed from the study. All Morris water maze trials were recorded and analyzed using the ANY-Maze tracking system (Stoelting, Chicago, IL).

Rotarod performance was evaluated using a Rotamex 4/8 (Columbus Instruments, Columbus, OH) machine following an accelerating rod protocol. The initial speed was set to 2 rpm with a linear acceleration to 40 rpm. Latency to fall was recorded as an index for locomotor ability at 4 trials per day with a 2–3 min inter-trial rest period.

2.4. Brain tissue section preparation and staining

Mice were anesthetized then transcardially perfused with PBS followed by 4% paraformaldehyde. Whole perfused brains were collected, post-fixed in 4% paraformaldehyde overnight at 4 °C, and then equilibrated in 30% sucrose in PBS for 3 days at 4 °C. Brains were sectioned at 16 μm using a cryostat (CM1850, Leica, Germany). For Nissl staining, sections were stained with 0.1% (w/v) cresyl violet for 5 min then dehydrated through graded ethanol rinses then cleared in xylene. Cresyl violet-stained sections were used to quantify cell number of the CA1 region of the hippocampus. Images were obtained with a 20× objective on a Zeiss Axio light microscope. Three different brain sections were quantified per mouse (n=3). Three CA1 region frames